Cross-Bridge Movement in Glycerinated Rabbit Psoas Muscle Fibers†

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ABSTRACT: The results of the present study demonstrate that the cross-linking properties of the S-1 and S-2 segments of myosin in glycerinated rabbit psoas muscle fibers are virtually identical with those observed in synthetic myosin filaments. Both systems show rapid cross-linking of the S-1 subunits to the thick filament surface at neutral pH and both exhibit a sharp decrease in the normalized rate constant ($k_{S-1}°$) of cross-linking S-1 to the filament surface over the range pH 7.4–8.0. The most probable explanation of these results (Sutoh K., et al. (1978) *Biochemistry 17*, 1234–1239) is that the myosin heads in both preparations are in close contact with the filament backbone at neutral pH but are dissociated from the backbone at alkaline pH as a result of electrostatic repulsion between the proximal surfaces. The S-1 cross-linking rate also decreases in the glycerinated fiber system at neutral pH and at very low ionic strength (~0.015 M). When E. Rome's earlier ((1967) *J. Mol. Biol. 27*, 591–602) X-ray measurements of the filament lattice dimensions of glycerinated fibers in high pH and neutral pH–low ionic strength buffers are combined with our cross-linking results, we estimate a minimum length of ~18.5 nm for the S-1 subunit. Although the actin-attached S-1 subunit in the glycerinated fibers moves out from the thick filament surface at alkaline pH, a major fraction of the S-2 region of myosin appears to remain close to the thick filament surface based on the similar cross-linking rates of the heavy and light meromyosin segments. A significant tendency for heavy meromyosin segments to associate with the thick filament backbone was also observed in ultracentrifuge studies of tryptic digests of synthetic myosin filaments.

In two previous studies (Sutoh & Harrington, 1977; Sutoh et al., 1978a), bifunctional cross-linkers were employed to probe the radial disposition of cross-bridges (myosin heads) in dispersed synthetic thick filaments and glycerinated myofibrils in rigor. We interpreted the results of these studies as indicating that neither the presence of Ca^{2+} ions nor the shift from resting to rigor state (at rest length) results in release of myosin heads from the thick filament surface at neutral pH and physiological ionic strength. The time course of immobilizing the myosin heads in synthetic thick filaments was, however, markedly sensitive to the pH of the solvent. On elevating the pH above neutrality, the normalized rate of cross-linking myosin heads to the thick filament surface (ratio of the rate of cross-linking heads to the rate of cross-linking rod segments) drops sharply over a narrow pH span (pH 7.4–8.0), suggesting that the heads of the myosin molecules move away from the thick filament surface at alkaline pH but are close to the surface at neutral pH. This interpretation is consistent with the depolarization measurements of Mendelson et al. (1973) and Mendelson & Cheung (1976) which have revealed that the rotational Brownian motion of myosin heads is highly restricted in synthetic filaments and relaxed glycerinated myofibrils at neutral pH as compared with the mobility of the heads of dispersed myosin molecules. A large increase in rotational mobility of the heads of synthetic filaments was observed by Mendelson & Cheung on raising the pH from 6.8 to 8.3 (μ = 0.12). Thomas et al. (1975) have reported similar changes in the mobility of the myosin heads with increasing pH based on electron paramagnetic resonance (EPR)[1] measurements.

Although our earlier cross-linking experiments with synthetic thick filaments indicated that the myosin heads (S-1 subunits) move away from the filament backbone at alkaline pH, a major fraction of the (long) S-2 tail of the heavy meromyosin (HMM) segment, which makes up about 50% of the myosin rod (Weeds & Pope, 1977; Highsmith et al., 1977; Sutoh et al., 1978b), appears to be close to the filament surface under these conditions just as it is at neutral pH. The time course of cross-linking HMM to the filament surface at pH 8.3 approximated that of the LMM segment of the rod which is firmly locked in the thick filament core (Sutoh et al., 1978). This is an important point since it suggests that, if there is a release of the myosin head from the thick filament surface in contracting muscle, then it may be a localized phenomenon involving the head segment and possibly only a small portion of the S-2 element near the head.

The purpose of the present study was to determine whether the same behavior was exhibited by the S-1 and S-2 segments of myosin in the native thick filaments of glycerinated rabbit psoas muscle. The contractile apparatus in such preparations remains intact and the glycerinated fibers show contraction in the presence of MgATP and activating concentrations of Ca^{2+} ion and generate as much tension as living muscle (Szent-Gyorgyi, 1949, 1951; Bendall, 1969). In dilute salt solutions, glycerinated muscle is in a rigid, inextensible condition in which it is believed that the S-1 subunits are attached in rigor linkage to their neighboring actin filaments (Rome, 1972a,b).

In this paper we show that the rate of cross-linking S-1 subunits to the thick filament surface in glycerinated rigor muscle fibers has a pH dependence closely similar to that observed in the synthetic thick filament systems, suggesting that the actin-attached myosin heads move away from the thick filament surface at alkaline pH. Outward movement of the myosin heads was also detected at neutral pH at very low ionic strength. Moreover, from a comparison of the cross-linking kinetics of the LMM and HMM regions of myosin, it appears that the S-2 segment remains close to the thick filament surface under these conditions. We have utilized this information in conjunction with the low-angle X-ray data

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[1] Abbreviations used: EPR, electron paramagnetic resonance; HMM and LMM, heavy and light meromyosin segment, respectively; DMS, dimethyl suberimidate; DTBP, dimethyl dithiobis(propionimidate); $NaDodSO_4$, sodium dodecyl sulfate.

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of Rome (1968) to estimate a minimum length for the S-1 subunit.

Materials and Methods

Reagents and Proteins. Dimethyl suberimidate (DMS) was purchased from Sigma; dimethyl dithiobis(propionimidate) (DTBP) and methyl acetimidate were from Pierce; α-chymotrypsin, trypsin, and soybean trypsin inhibitor were obtained from Worthington Biochemical and were used without further purification. Glycerinated rabbit psoas muscle bundles were prepared following the procedure of Rome (1967). These were stored in 50% glycerol, 50% water, 6.67 mM potassium phosphate buffer (pH 7.0) at −20 °C for at least 3 weeks before use. All fiber bundles employed in the present study were glycerinated at the resting length of the psoas muscle. The sarcomere length of the glycerinated fibers was determined from measurements of the light diffraction pattern using a helium–neon gas laser (Model HN-10, λ = 628 nm and 1.3 mm diameter of beam) as the source of parallel, monochromatic light. The sarcomere length of 15 fiber preparations in rigor was 2.7 ± 0.1 μm. Myofibrils were prepared according to Kundrat & Pepe (1971). The approximate concentration of myofibrils was determined by dissolving them in 5% sodium dodecyl sulfate ($NaDodSO_4$) and measuring OD_{280}, assuming $E_{280}^{1\%}$ = 7.0 (Sutoh & Harrington, 1977).

Cross-Linking and Proteolytic Digestion of Glycerinated Muscles. All cross-linking reactions were performed at 5 °C. Ca. 0.1–0.15 g (wet weight) of 1-mm-diameter (3-cm-long) glycerinated muscle bundles was reacted with DMS for up to 4 h at pH 7.0–8.5. The concentration of cross-linker was gradually reduced from 5.5 mM at pH 7.0 to 1.8 mM at pH 8.5 since the rate of amidination of lysine side chains has a positive pH dependence (Hunter & Ludwig, 1962). According to Rome (1968) the filament lattice spacing (lateral distance between filaments) of glycerinated muscle fibers is a function of the pH and ionic strength of the interfilament medium. To ensure a lattice spacing sufficiently large to prevent cross-linking of the myosin heads at alkaline pH, we used a low ionic strength solvent (~0.03 M) in the present study. In the pH range 7.0–7.4, the solvent consisted of 8 mM NaCl and 40 mM imidazole hydrochloride; in the pH range 7.4–8.5, the solvent consisted of 8 mM NaCl and 40 mM triethanolamine hydrochloride. We used both imidazole and triethanolamine buffers at pH 7.4 and observed no significant difference in the relative rate of cross-linking myosin heads to rod segments. The cross-linking reaction was stopped at various times by washing the muscle fibers three times with 75 mL of 40 mM sodium phosphate (pH 6.2) for a period of 30 min (10 min for each wash). Then the cross-linked fibers were homogenized in a Sorvall Micro-Omnimixer for 45 s at 60 V in 80 mM NaCl and 40 mM imidazole hydrochloride buffer (pH 7.0). Proteolysis of these myofibrils (~3 mg/mL) at various stages of cross-linking was carried out in the presence of EDTA (0.1 volume of 0.1 M EDTA) using α-chymotrypsin (0.3 mg/mL) for 15 min at 20 °C. Digestion was terminated by addition of phenylmethanesulfonyl fluoride (final concentration, 6 mM). The kinetics of cross-linking the head and rod segments of myosin was determined from densitometry of the head and rod bands observed in electrophoresis in $NaDodSO_4$–polyacrylamide gels (Sutoh & Harrington, 1977). Since the rate of amidination reaction between lysine side chains and imidate esters of DMS varies with pH (Hunter & Ludwig, 1962), the rate constants for cross-linking the S-1 subunits and rod segment, $k_{S\text{-}1}$ and k_{rod}, are expected to show pH dependence even if the radial disposition of myosin heads remains unchanged with pH. To eliminate this factor, which is unrelated to structural changes in the filament, the ratio of the two rate constants, $k_{S\text{-}1}/k_{rod}$ ($=k_{S\text{-}1}°$), has been determined at each pH.

Cross-Linking of Glycerinated Myofibrils. All cross-linking and digestion procedures were carried out at 5 °C. Glycerinated myofibrils, 3 mg/mL, suspended in 80 mM NaCl and 40 mM imidazole hydrochloride (pH 7.4) were cross-linked by DMS or dimethyl dithiobis(propionimidate) (DTBP) up to 4 h. At various times the cross-linking reaction was quenched by addition of 0.1 volume of 1 M lysine (pH 7.8). When the cross-linked myofibrils were digested by α-chymotrypsin, the cross-linking reaction was quenched after digestion. The digestion was carried out with 0.3 mg/mL of α-chymotrypsin for 20 min in the presence of EDTA (0.1 volume of 0.1 M EDTA, pH 7.0) and then terminated by phenylmethanesulfonyl fluoride (final concentration 6 mM).

Kinetics of Cross-Linking the HMM Heavy Chain at pH 8.3 in Glycerinated Muscle Fibers. Glycerinated muscle fibers were cross-linked with DMS (2.2 mM) in 8 mM NaCl and 40 mM triethanolamine hydrochloride (pH 8.3) as described earlier. The time courses of cross-linking HMM and LMM were obtained by digesting the cross-linked muscle fibers in 80 mM NaCl, 40 mM imidazole hydrochloride, and 1 mM $CaCl_2$ (pH 7.0) with 0.3 mg/mL of α-chymotrypsin for 80 min at 20 °C. Densitometry of $NaDodSO_4$–polyacrylamide gels of the resulting product showed that in un-cross-linked fibers the digestion of the myosin heavy chain was ~95% complete. To check the effect of cross-linking on the formation of HMM and LMM by chymotryptic digestion, a cleavable cross-linker, DTBP, was used to carry out the same reaction as above. Since the disulfide bond is expected to be unstable at alkaline pH we used a neutral pH buffer consisting of 8 mM NaCl and 40 mM imidazole hydrochloride for the cross-linking reaction. Cross-linked protein samples were dialyzed against 0.1% $NaDodSO_4$, 10 mM Tris-HCl, and 2% β-mercaptoethanol (pH 8.0) for 10 days at room temperature to cleave the disulfide bonds of the cross-links. The $NaDodSO_4$–polyacrylamide gel patterns of digested proteins with and without cross-linking reaction were then compared by densitometry.

The cross-linking procedure was also carried out at 5 °C with 1.5 mM DMS in 10 mM sodium borate (pH 9.0), and the time course of cross-linking the HMM and LMM segments was obtained using the same procedure as described above.

Procedure for Comparing Kinetics of Cross-Linking in Synthetic Filaments and Glycerinated Fibers. The amounts of the intact heavy chain and the rod, S-1, LMM and HMM fragments of myosin, determined by densitometry of $NaDodSO_4$–polyacrylamide gels of both fibers and synthetic thick filaments, all showed single exponential decays with time of cross-linking. However, the overall rate of cross-linking in the fibers was lower than in the filaments, presumably because of slower diffusion of the reagent into the fibers. To compare the kinetics of the cross-linking in the fiber and filament systems, we therefore multiplied the exponential constants for the fiber data by a factor (2.5) equal to the ratio of the rate constants for the decay of the intact heavy chains of the filament and fiber. This enabled both sets of data to be compared in a single figure. It should be emphasized that the rates of cross-linking the various segments of myosin are *apparent* rates since any portion of myosin which has undergone $n \geq 1$ cross-linking reactions will give the same drop in percent intensity on $NaDodSO_4$–polyacrylamide gels.

Tryptic Digestion of Synthetic Thick Filaments. Synthetic thick filaments (1 mg/mL) were formed at pH 8.3 according

to Josephs & Harrington (1966) and digested with 0.02 mg/mL of trypsin in 80 mM NaCl, 40 mM triethanolamine hydrochloride and 1 mM $CaCl_2$ (pH 8.3) for 8 min at 5 °C. Soybean trypsin inhibitor (0.04 mg/mL) was added to stop the digestion. Electrophoresis of this mixture on $NaDodSO_4$–polyacrylamide gels showed that tryptic digestion is complete and that HMM and LMM are the major products under these conditions. Electron micrographs of samples of these preparations negatively stained with uranyl acetate showed that the myosin filaments remain intact after tryptic digestion.

Trypsin rather than chymotrypsin was used to digest the synthetic filaments since the HMM formed from chymotryptic digestion under these solvent conditions showed a complex banding pattern (presence of internal clips) on $NaDodSO_4$–polyacrylamide gels.

Sodium Dodecyl Sulfate Gel Electrophoresis. $NaDodSO_4$ gel electrophoresis was performed as previously described (Sutoh et al., 1978a). Gels of 5% acrylamide and 0.13% bis(acrylamide) were used. Since densitometry measurements of the gel patterns showed that the intensity of the actin band remained invariant during the cross-linking reaction with DMS or DTBP, this band was used as an internal standard in all of the kinetic analyses. No significant differences were observed in the $NaDodSO_4$ gel electrophoresis pattern of the cross-linked glycerinated fibers analyzed in the present study and the myofibrils reported earlier (Sutoh & Harrington, 1977).

Sedimentation Velocity Studies. A Spinco Model E ultracentrifuge was employed at 15 000 rpm at a rotor temperature of 5 °C. Thirty-millimeter path length synthetic boundary cells were used. Schlieren peak areas were obtained by tracing the enlarged Schlieren patterns on heavy-weight paper; then each peak was cut out and weighed.

Results and Discussion

Glycerinated muscle preparations were cross-linked with DMS in dilute salt solutions buffered at different pH values over the range pH 7.0–8.5. At various stages of the reaction, excess reagent was removed from aliquots of the reacting system which were then digested with chymotrypsin in the presence of EDTA to cleave the head-rod linkage (see Weeds & Taylor, 1975; Bagshaw, 1977; Weeds & Pope, 1977). The extent of cross-linking S-1 and rod segments of myosin was determined by densitometry of the bands corresponding to these species following electrophoresis on $NaDodSO_4$–polyacrylamide gels as described in Materials and Methods. As in the case of cross-linking synthetic filaments (Sutoh et al., 1978a), the time course of cross-linking each of these segments within the thick filaments of the glycerinated muscle followed first-order kinetics at all pH values examined. Thus, even though a major fraction of the myosin heads is attached to neighboring thin filaments (Rome, 1972a), the heads appear to be equivalent in their apparent rates of cross-linking to the thick filament surface in the rigor muscle. The ratio of the rate constant for cross-linking S-1 to that of rod, $k_{S\text{-}1}°$ (= $k_{S\text{-}1}/k_{rod}$), is plotted vs. pH in Figure 1 where it will be seen that the titration curve for the muscle fiber is closely similar to that observed earlier for the synthetic filaments. In both preparations the rates of cross-linking myosin heads to the thick filament surface remains unchanged on elevating the pH to about 7.4, but $k_{S\text{-}1}°$ shows a sharp decrease over a narrow pH range (7.4–8.0) and levels off at a value of ~0.04 above pH 8.0.

We have argued (Sutoh et al., 1978a) that the most likely reason for the rapid drop in $k_{S\text{-}1}°$ observed in the synthetic

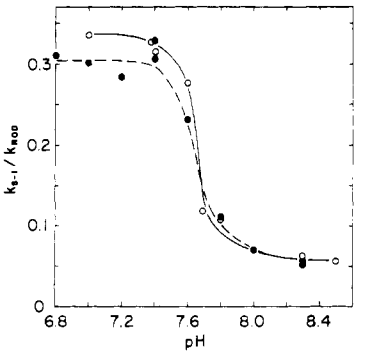

FIGURE 1: Titration curves of the normalized rate of cross-linking myosin heads $k_{S\text{-}1}°$ ($=k_{S\text{-}1}/k_{rod}$). (a) In glycerinated muscle fibers (O—O). Cross-linking was carried out in 8 mM NaCl and 40 mM imidazole hydrochloride (pH 7.0–7.4); 8 mM NaCl and 40 mM triethanolamine hydrochloride (pH 7.4–8.5). (b) In synthetic thick filaments (●---●) (from Sutoh et al., 1978a). Cross-linking was carried out in 80 mM KCl, 40 mM imidazole hydrochloride, and 0.1 mM $MgPP_i$ (pH 6.8–7.4); 80 mM KCl and 40 mM triethanolamine hydrochloride (pH 7.4–8.3). Cross-linker, dimethyl suberimidate; temperature, 5 °C.

thick filament system is outward movement of the S-1 subunits from the filament surface. The same interpretation is given the $k_{S\text{-}1}°$ vs. pH profile of the actin-attached myosin heads in the glycerinated muscle fiber. The similarity of the titration curves in Figure 1 indicates that this process has a common origin in the two systems and may be due to electrostatic repulsion between the S-1 subunits and the filament backbone resulting from titration of ionizing side groups (possibly histidine residues) over the range pH 6.8–8.3.

Measurements of the equatorial reflections (Rome 1967, 1968) of low-angle X-ray diffraction patterns of glycerinated rabbit psoas muscle immersed in dilute salt solution indicate that the double hexagonal lattice of thick and thin filaments expands reversibly on elevating the pH. A comparable expansion of the lattice was observed at *neutral* pH on lowering the ionic strength well below 0.10 M. Rome (1967, 1968) has proposed that the reversible expansion in the filament lattice with pH and ionic strength results from a shift in the balance between long-range electrostatic forces and van der Waals attractive forces. According to her X-ray measurements, the myosin-to-actin center-to-center separation of filaments in glycerinated rabbit psoas muscle in rigor increases approximately linearly with the pH of the medium. In fibers glycerinated at rest length (2.8 ± 0.2 μm sarcomere length), this spacing increases about 4.5 nm (from 19.5 to 24.0 nm) on elevating the pH from 4.0 to 9.0 at an ionic strength of 0.10 M. This is equivalent to an increase in the myosin-to-actin spacing of about 0.9 nm per pH unit. Although the solvents employed in the present study were of lower ionic strength (~0.03 M), we expect about the same magnitude in lateral expansion over this pH range based on Rome's (1967) experiments on the effect of ionic strength on the filament lattice dimensions. Accordingly, an expansion of the myosin-to-actin spacing of only about 0.5 nm occurs over the range (pH 7.4–8.0) where the major change in the $k_{S\text{-}1}°$ vs. pH plot is observed (Figure 1). Thus it appears that this relatively small outward movement of the myosin heads from the filament surface, though it gives a dramatic change in the rate of cross-linking the actin-attached S-1 subunits, has little measurable effect on the overall lattice dimensions.

Kinetics of Cross-Linking HMM Segments to the Thick Filament Surface in Glycerinated Fibers. In our study of synthetic thick filaments (Sutoh et al., 1978a), the rate of cross-linking the HMM segment of myosin to the thick fi-

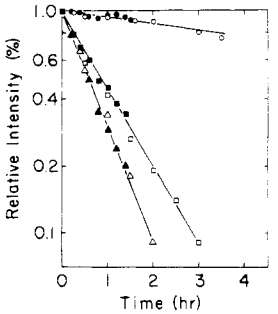

FIGURE 2: Relative rates of cross-linking S-1 (○, ●), HMM (□, ■) and LMM (△, ▲) at pH 8.3 and 5 °C in synthetic thick filaments (○, □, △) (Sutoh et al., 1978a) and glycerinated muscle fibers (●, ■, ▲) (present study). The data are normalized with respect to myosin heavy chain (see Materials and Methods). Cross-linking conditions: myosin filaments, 1 mg/mL in 80 mM KCl and 40 mM triethanolamine hydrochloride; glycerinated fibers, 3 mg/mL in 8 mM NaCl and 40 mM triethanolamine hydrochloride; cross-linker, dimethyl suberimidate, 1.8 mM.

lament surface at pH 8.3 was about the same as that of LMM, suggesting that a significant portion of the S-2 tail of HMM is close to the filament surface under conditions where the myosin heads are displaced away from the surface. This experiment has now been repeated on glycerinated muscle fiber preparations and the results are shown in Figure 2 which presents plots of log relative intensity vs. time of cross-linking for S-1, HMM, and LMM derived from densitometry measurements of $NaDodSO_4$–polyacrylamide gels. To compare the data obtained on synthetic thick filaments and glycerinated fibers, the decay curve of each fragment was normalized as described in Materials and Methods. In the present study, glycerinated fiber preparations at various times of cross-linking were digested with chymotrypsin in the presence of EDTA to form S-1 or in the presence of Ca ions to cleave the protease-sensitive LMM–HMM site (Bagshaw, 1977; Weeds & Pope, 1977; see Materials and Methods). To show that modification of the lysine side chains does not influence the subsequent proteolytic digestion, we have also used the cleavable reagent DTBP to cross-link glycerinated fibers. The $NaDodSO_4$–polyacrylamide gel patterns of digested proteins with and without cross-linking were indistinguishable after complete cleavage of the cross-linker disulfide bonds. Thus the rate of immobilization of each myosin segment can be determined from the time-dependent fall in intensity of each corresponding band on $NaDodSO_4$ gels. It is clear from Figure 2 that the normalized rates of cross-linking of the S-1, LMM, and HMM segments of myosin are the same in both the synthetic filaments and the native thick filaments of the glycerinated fiber preparations, and, moreover, the rate of cross-linking HMM to the thick filament surface at pH 8.3 is comparable to that of LMM and very much faster than the rate of cross-linking myosin heads. These observations, like those of Figure 1, indicate that synthetic thick filaments are a good analogue of native thick filaments. If we assume (1) that the lysine residues are randomly distributed over the entire rod surface and (2) that the S-2 segment comprises about 50% of the myosin rod (Weeds & Pope, 1977; Sutoh et al., 1978b), a major fraction of this segment must be close to the thick filament backbone. At pH 9.0 and 0.01 M ionic strength, the cross-linking of HMM and LMM in glycerinated fibers also showed that the rate of cross-linking HMM is comparable to that of LMM (k_{HMM} = 0.27 h^{-1}; k_{LMM} = 0.38 h^{-1}). Therefore, the major portion of S-2 is part of the thick filament surface even though the myosin-to-actin lattice spacing increases about 2.0 nm over the pH range 7.0–9.0. Since the myosin heads are presumed to be attached to neighboring actin filaments in rigor linkage in the glycerinated fiber, the increase in the myosin-to-actin lattice spacing provides a rather precise measure of the outward radial movement of the heads on elevating the pH. At pH 9 the myosin-to-actin spacing of glycerinated rabbit psoas fiber (~24.0 nm at 0.1 M ionic strength) approximates the spacing observed in fully contracted living muscle (Rome, 1972a; Huxley, 1968). Yet, judging from the similar cross-linking rates of HMM and LMM at this pH (μ = 0.01), a major fraction of the S-2 element must be held close to the filament surface just as it is at pH 7.0. Thus, as we have suggested earlier (Sutoh et al., 1978a), only release of the S-1 subunit and possibly a small region of the S-2 segment near the S-1/S-2 junction may be required to accommodate the variable lattice spacings as the sarcomere shortens in actively contracting muscle.

Ultracentrifuge Experiments Showing Binding of S-2 to the Thick Filament Surface. Additional evidence supporting the idea that the S-2 portion of HMM is associated with the filament backbone was obtained from the following experiments. When synthetic thick filaments were digested with trypsin in the presence of Ca ions at pH 8.3 to form HMM with a long S-2 tail (Harrington et al., 1978), the HMM showed a strong tendency to associate with the filament backbone. Velocity sedimentation patterns of the digested filament at pH 8.3 in 80 mM NaCl, 40 mM triethanolamine hydrochloride exhibit two Schlieren peaks. The slower peak was identified as HMM from its sedimentation coefficient ($s_{20,w}$ = 5 S) and the faster, hypersharp peak as the residual filament. This identification was confirmed by $NaDodSO_4$ gel electrophoresis of the supernatant and pellet fractions obtained after the fast boundary had moved to the base of the centrifuge cell. Only HMM was detected in the supernatant solution, whereas the pellet contained both HMM and LMM. Based on area measurements of the Schlieren boundaries, about 50% of the tryptic HMM formed on digestion is still associated with the filament at pH 8.3 ($K_{assoc} \sim 9 \times 10^5$ M^{-1}). On the other hand, when the digest was dialyzed at pH 7.0 in 80 mM NaCl, 40 mM imidazole hydrocloride and then examined in the ultracentrifuge, about 90% of the HMM was associated with the faster sedimenting filament peak judging from the shift in areas of the bimodel pattern ($K_{assoc} \sim 3 \times 10^7$ M^{-1}).

The pH-sensitive association of HMM with the filament backbone appears to be completely reversible. When the pH of the buffering solvent was cycled by dialysis, viz., pH 8.3 to 7.0 to 8.3, the bimodal velocity sedimentation pattern of the original digest was restored. Since tryptic HMM has about the same molecular weight as chymotryptic HMM (based on comigration of their heavy chain bands on $NaDodSO_4$ gel electrophoresis), these results clearly indicate a strong tendency for the long S-2 segment of myosin to bind to the thick filament backbone and therefore support our contention that only the S-1 subunits are released from the thick filament backbone at alkaline pH.

Although the HMM fragment shows a decreased binding affinity for the thick filament surface at alkaline pH, our cross-linking studies indicate that a major fraction of the S-2 region in an undigested myosin molecule remains close to the filament surface. This difference in behavior is probably due to the presence of an intact S-2-LMM junction in myosin which serves as an additional restraint to outward radial movement of the HMM segment.

When S-2 is prepared by chymotryptic digestion of HMM in the presence of EDTA (Weeds & Pope, 1977), the resulting

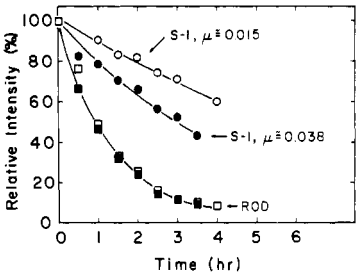

FIGURE 3: Time course of cross-linking the S-1 and rod segments in glycerinated muscle fibers at pH 7.0. Cross-linking was carried out in 8 mM NaCl and 40 mM imidazole hydrochloride (●, ■) or 20 mM imidazole hydrochloride (○, □). The relative intensities of the S-1 band (○, ●) and the rod band (□, ■) were determined by densitometry on $NaDodSO_4$ gels of cross-linked myofibrils digested by chymotrypsin (0.3 mg/mL) in the absence of divalent cations. Cross-linking conditions: total protein concentration, 3 mg/mL; cross-linker, DTBP, 3.5 mM; temperature, 5 °C.

fragment has a molecular weight of about 100 000 (Sutoh et al., 1978b) and shows a marked tendency to undergo self-association unlike the shorter S-2 (mol wt 60 000–80 000) isolated by earlier workers (Lowey et al., 1969; Biro et al., 1972; Burke et al., 1973; Goodno et al., 1976). This particle appears to have a sticky region of about 20.0–25.0-nm length near the LMM–HMM junction which could serve to hold the S-2 segment of myosin down onto the thick filament surface (Sutoh et al., 1978b). The sticky region may be responsible for the association of HMM (with long S-2 tail) with the filament backbone observed in the present study.

Minimum Length of the S-1 Subunit. According to the X-ray measurements of Rome (1967), the center-to-center distance (myosin-to-actin) of the filament lattice of glycerinated rabbit psoas muscle in rigor expands about 1.6 nm in neutral pH buffers on lowering the ionic strength from 0.11 to 0.01 M. Since this is about the same increase in filament separation as that observed over the range pH 7–8.5 at physiological ionic strength, it should be reflected in a drop in the S-1 cross-linking rate if the actin-attached heads move away from the thick filament surface. At pH 7 the normalized rate of cross-linking myosin heads, $k_{S\text{-}1}°$ (= $k_{S\text{-}1}/k_{rod}$), to synthetic filaments and glycerinated myofibrils is about 0.34 (Sutoh & Harrington, 1977) and this ratio remains unchanged down to an ionic strength of 0.03 M in the glycerinated fiber system (see Figure 1). When the ionic strength is lowered to 0.015 M, however, we find a significant decrease in $k_{S\text{-}1}°$. As shown in Figure 3, $k_{S\text{-}1}°$ falls to a value of 0.17. This is about the midpoint of the pH-titration curve (Figure 1) where $k_{S\text{-}1}°$ levels off at a value of 0.04 when all of the myosin heads leave the thick filament surface. At this ionic strength (~0.015 M), a major portion of the S-2 element must still bind to the thick filament backbone since the time course of cross-linking the rod segments of myosin is essentially the same as that observed in the high ionic strength solvents (see Figure 3). Thus taking into account both the pH titration data of Figure 1 and the ionic strength measurements at neutral pH, it appears that the S-1 subunits detach and move away from the thick filament surface at a myosin–actin filament spacing near 24.0 nm. The minimum length of the S-1 subunit required to span this gap is about 18.5 nm assuming that the myosin heads are attached to the actin filament at the rigor linkage angle (45°) and using diameters of 14.0 and 8.0 nm, respectively, for the myosin (core) and actin filaments (Page & Huxley, 1963). The minimum length of the S-1 subunit inferred from the cross-linking data is appreciably greater than estimates of this parameter based on X-ray scattering measurements of the radius of gyration of S-1 assuming a prolate ellipsoid of revolution (12.0–15.0 nm; Kretzschmar et al., 1976, 1978) or from image reconstruction studies of the acto-S-1 complex (12.0 nm; Moore et al., 1970), but it is in reasonable accord with recent electron microscope studies of dispersed myosin molecules (19.0 nm, Offer & Elliott, 1978; 21.0 nm, Takahashi, 1978).

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