Effect of pH on the Cross-Bridge Arrangement in Synthetic Myosin Filaments†

Kazuo Sutoh, Yu-Chih Chen Chiao, and William F. Harrington*

ABSTRACT: Synthetic thick filaments were cross-linked with dimethyl suberimidate at various pH values over the range pH 6.8–8.3. The rate of cross-linking myosin heads to the thick filament surface decreases significantly over a narrow pH range (7.4–8.0) despite the fact that the rate of the chemical reaction (amidination of lysine side chains) shows a positive pH dependence. The fall in rate cannot be ascribed to dissociation of the filament during the cross-linking reaction since the sedimentation boundary of the cross-linked filament (pH 8.3) remains unaltered in the presence of high salt (0.5 M). The decreased rate of cross-linking is also not caused by a shift in reactivity of a small number of highly reactive lysine groups, since the time course of cross-linking (pH 7.2) is unaffected by preincubation with a monofunctional imidate ester. Our results suggest that the heads of the myosin molecules move away from the thick filament surface at alkaline pH but are held close to the surface at neutral pH.

In a previous study (Sutoh & Harrington, 1977), we employed bifunctional cross-linking reagents to investigate the radial disposition of cross-bridges (myosin heads) in dispersed synthetic thick filaments and glycerinated myofibrils in rigor. We concluded from these experiments that neither the presence of Ca ions nor the shift from resting to rigor state (at rest length and at neutral pH) results in release of myosin heads from the thick filament surface.

In the present study, the kinetics of cross-linking myosin heads to the thick filament surface has been extended to the alkaline pH range (up to pH 8.3). 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). This observation when taken in conjunction with recent experiments (Mendelson & Cheung, 1976; Thomas et al., 1975) showing that the rotational mobility of myosin heads in thick filaments, as measured by depolarization of fluorescence or EPR spectroscopy, increases with increasing pH strongly suggests that myosin heads are in close contact with the filament surface at neutral pH (pH 6.8–7.4), but are released from the surface on raising the pH.

Materials and Methods

Reagents and Proteins. Dimethyl suberimidate (DMS)[1] and methyl acetimidate were purchased from Pierce; chymotrypsin and papain were from Worthington Biochemical. Heavy meromyosin (HMM) and subfragment 1 (S-1) were prepared according to Weeds & Taylor (1975) using chymotrypsin. Subfragment 2 (S-2) was prepared as follows: HMM (3 mg/mL) prepared by chymotryptic digestion (Weeds & Taylor, 1975) was again digested by chymotrypsin (0.02 mg/mL) in 0.1 M NaCl, 20 mM phosphate, and 2 mM EDTA (pH 7.0) for 10 min at 25 °C. After addition of 3 volumes of ethanol, the solution was stirred for 2 h at room temperature and the resulting precipitate collected by centrifugation and suspended in 20 mM phosphate (pH 6.2). Following exhaustive dialysis against 20 mM phosphate (pH 6.2), the solution was centrifuged at 100 000*g* (1 h) to remove aggregated materials and then dialyzed against 20 mM phosphate (pH 5.7), and the precipitate was again collected by centrifugation. The precipitate was dissolved in an appropriate buffer for use. This S-2 preparation shows a major component of 60 000 on NaDodSO$_4$ gel electrophoresis. The molecular weight is significantly higher than that reported earlier for subfragment 2 (37 000) prepared by either papain or tryptic digestion (Lowey et al., 1969; Biro et al., 1972; Balint et al., 1975) but is in good agreement with the value reported for the recent preparation of S-2 by Weeds & Pope (1977) obtained from chymotryptic digestion of HMM in the absence of divalent metals. A detailed report of the molecular properties of our S-2 preparation will appear elsewhere.

Cross-linking and Proteolytic Digestion of Synthetic Thick Filaments. A. The Effect of pH on the Cross-linking Reaction. Cross-linking and chymotryptic digestion of the synthetic thick filaments followed procedures described in the preceding paper (Sutoh & Harrington, 1977) except that dimethyl suberimidate (DMS) was used as cross-linking reagent. All procedures were carried out at 4 °C. Synthetic thick filaments (1 mg/mL), prepared according to Josephs & Harrington (1966) at pH 8.3, were adjusted to lower pH by dialysis. In the pH range 6.8–7.4, the solvent consisted of 80 mM KCl, 40 mM imidazole and 0.1 mM magnesium pyrophosphate (Mg-PP$_i$); in the pH range 7.4–8.3 the solvent consisted of 80 mM KCl and 40 mM triethanolamine. We used both the imidazole buffer and the Tris buffer at pH 7.4 and observed no significant difference in the rate of cross-linking the myosin heads (see Figure 2). Magnesium pyrophosphate was added to eliminate any possible association of filaments in the neutral pH range. Previous studies (Sutoh & Harrington, 1977) have shown that this ligand has no effect on the time course of cross-linking myosin heads in the range pH 6.8–7.4. DMS, dissolved in the appropriate buffer, was added to the filament solution to start each cross-linking reaction. The concentration of cross-linker was gradually reduced with increasing pH since the rate of the amidination reaction of lysine side chains has a positive pH dependence (Hunter & Ludwig, 1962). In the present study,

† Contribution No. 945 from the Department of Biology, McCollum-Pratt Institute, The Johns Hopkins University, Baltimore, Maryland 21218. *Received August 16, 1977.* This investigation was supported by U.S. Public Health Service Grant AM-04349.

[1] Abbreviations used: DMS, dimethyl suberimidate; DTBP, dimethyl dithiobispropionimidate; IM-SH, methyl mercaptobutyrimidate; HMM, heavy meromyosin; LMM, light meromyosin; S-1, subfragment 1; S-2, subfragment 2; NaDodSO$_4$, sodium dodecyl sulfate.

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the concentration of the cross-linker was varied between 2.0 and 0.6 mg/mL depending on pH to give approximately equivalent levels of cross-linking after 6 h. The cross-linking reaction was allowed to proceed for different periods of time (up to 6 h), aliquots of filament solution were removed, 0.1 volume of 100 mM EDTA (pH 7.0) was added to each, and the partially cross-linked filaments were digested by chymotrypsin (0.3 mg/mL) for 10 min. Proteolysis was terminated by addition of phenylmethanesulfonyl fluoride (final concentration 1 mM). The cross-linking reaction was quenched by addition of 0.1 volume of 2 M ethanolamine (pH 8.0) immediately after chymotryptic digestion. In some cases, ethanolamine was added to aliquots of cross-linked but undigested filaments to determine the extent of cross-linking of myosin heavy chains.

B. Cross-linking of Synthetic Thick Filaments after Prior Modification with Methyl Acetimidate. All procedures were carried out at 4 °C. Synthetic thick filaments formed at pH 8.3 (Josephs & Harrington, 1966) were dialyzed against solvent consisting of 80 mM KCl, 40 mM imidazole, and 0.1 mM Mg-PP_i (pH 7.2). Methyl acetimidate (0.1 mg/mL) was added to the resulting filament solution and the amidination reaction between lysine side chains and methyl acetimidate allowed to proceed. After 2 h of reaction with the monofunctional reagent, DMS (1.5 mg/mL) was added to start the cross-linking reaction. At the same time, DMS was also added to control filament solution without any previous treatment. At various reaction times aliquots were removed and treated with chymotrypsin and the cross-linking reaction was terminated as described in A.

The extent of amidination of lysine side chains by methyl acetimidate was determined by titrating free amino groups of myosin with trinitrobenzenesulfonic acid (Habeeb, 1966). The number of lysine chains in myosin blocked with the monofunctional reagent was about 10 and 30 after 2 h and 6 h of reaction, respectively.

C. Kinetics of Cross-linking of Heavy Meromyosin Heavy Chain at pH 8.3. All procedures were carried out at 4 °C. Synthetic thick filaments formed as in A were cross-linked with DMS (0.6 mg/mL) in 80 mM KCl and 40 mM triethanolamine (pH 8.3). Aliquots of cross-linked filament solution were removed at different reaction times and 100 mM $CaCl_2$ added to each to give a final concentration of 1 mM. The filaments were then digested with chymotrypsin (0.3 mg/mL) for 10 min. The proteolysis and cross-linking reactions were terminated as described above. A known amount of actin, purified from acetone powder, was added as a standard protein before denaturation with $NaDodSO_4$.

Cross-linking and Proteolytic Digestion of HMM. HMM (1 mg/mL) in 80 mM NaCl and 40 mM imidazole (pH 7.4) was cross-linked with dimethyl suberimidate (1.2 mg/mL) for 2 h at 4 °C. About 50% of the myosin heads are cross-linked in the synthetic thick filament system under these conditions. Cross-linked and non-cross-linked HMM preparations were dialyzed against 0.1 M KCl, 20 mM phosphate, and 2 mM EDTA (pH 6.9) after quenching the cross-linking reaction with 0.1 volume of 1 M lysine (pH 7.0). The resulting HMM preparations were digested with 0.02 mg/mL chymotrypsin for 15 min at room temperature and proteolysis was terminated as described in A: $NaDodSO_4$ gel electrophoresis of this proteolytic product showed that 90–95% of HMM is digested to subfragment 1 (S-1) and subfragment 2 (S-2). The sum of the intensities of the S-1 band (90 000) and the S-2 band (60 000) in the $NaDodSO_4$ gel was about equivalent to the intensity of the HMM band (150 000) lost by proteolysis, indicating insignificant internal cleavage in the S-1 and S-2 particles.

Kinetics of Cross-linking Various Segments of Myosin. The following procedure was used to follow the time-course of cross-linking myosin heavy chain, rod, and subfragment 1.

Densitometer recordings of the bands corresponding to these components on $NaDodSO_4$ gels were traced on heavy weight paper and each peak was cut out and weighed. All peak areas were normalized using the peak area of the standard protein (catalase or actin) in each gel to avoid errors which may stem from fluctuations in the amount of protein loaded on the gels. Gels loaded with non-cross-linked samples (with or without proteolytic digestion) were chosen as standard gels. The intensity of each band on gels of cross-linked samples was expressed as "relative intensity (%)", setting the relative intensity of the corresponding band on the standard gel as 100%. Thus, the relative intensity of any migrating band converges to 100% at zero cross-linking time regardless of its absolute intensity (see Figure 1).

The apparent first-order rate constants of cross-linking myosin heads ($k_{S\text{-}1}$) and myosin rods (k_{rod}) at each pH were determined by fitting the relative intensity vs. time plots for each of these fragments to a single exponential curve using the least-squares method (Figure 1).

$NaDodSO_4$ gel electrophoresis was carried out according to Weber & Osborn (1969) employing a Tris-HCl buffer system. All samples were denatured by adding 0.25 volume of 4% $NaDodSO_4$, incubated at 37 °C overnight, and then dialyzed against 10 mM Tris, 0.1% $NaDodSO_4$, and 0.2% 2-mercaptoethanol (pH 8.3) before electrophoresis. A known amount of catalase or actin was added as a standard protein just before denaturing the samples.

Gels with 5% acrylamide and 0.13% bis(acrylamide) or 0.5% agarose, 2% acrylamide, and 0.1% bis(acrylamide) were routinely used. To identify molecular species appearing on the bands in $NaDodSO_4$ gels, purified HMM, S-1, S-2, and myosin rod were coelectrophoresed with samples. The molecular weights of HMM, S-1, and S-2 were estimated on 5% acrylamide gels using myosin heavy chain, C protein, paramyosin, phosphorylase, bovine serum albumin, catalase, and actin as standard marker proteins (Weber & Osborn, 1969).

Sedimentation velocity experiments of synthetic thick filaments were carried out following the procedure of Josephs & Harrington (1966) using the synthetic boundary cell. Rotor speed was 12 000 rpm.

Results

pH Dependence of Cross-linking Heads and Rod Segments in the Myosin Synthetic Thick Filament. In the present study, dimethyl suberimidate (DMS) was used to cross-link the synthetic thick filament in place of dimethyl dithiobispropionimidate (DTBP) or methyl mercaptobutyrimidate (IMSH) (which were used in our earlier work (Sutoh & Harrington, 1977)), since the disulfide bond is expected to be unstable at alkaline pH. Considering the structural similarity between DMS and DTBP (two CH_2 groups in DMS replace the S-S bond in DTBP (Davis & Stark, 1970; Wang & Richards, 1974)), the time course of cross-linking various segments of myosin with DMS is expected to be closely similiar to that obtained with DTBP. Indeed, we observed that the (normalized) time courses of cross-linking various myosin segments of the thick filament with these two reagents were virtually indistinguishable at neutral pH.

The time course of cross-linking both the head segment (S-1) and rod segment of myosin in synthetic thick filaments using dimethyl suberimidate as cross-linker follows first-order kinetics at all pH values examined over the range 6.8–8.3. Synthetic filaments were cross-linked for various periods of time

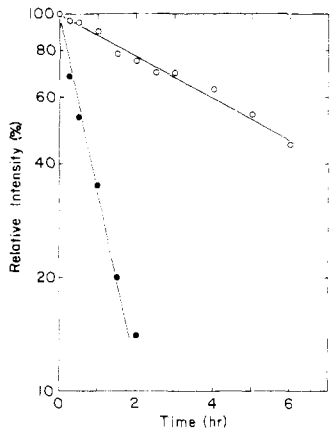

FIGURE 1: Time course of cross-linking the head and rod segments in synthetic thick filaments. The relative intensities of the rod band (●) and the S-1 band (○) were determined by densitometry on $NaDodSO_4$ gels of cross-linked filament digested by chymotrypsin in the absence of divalent cations. Cross-linking conditions: solvent, 80 mM KCl and 40 mM triethanolamine (pH 7.8); myosin filament, 1 mg/mL; cross-linker, dimethyl suberimidate (1.1 mg/mL); temperature, 4 °C.

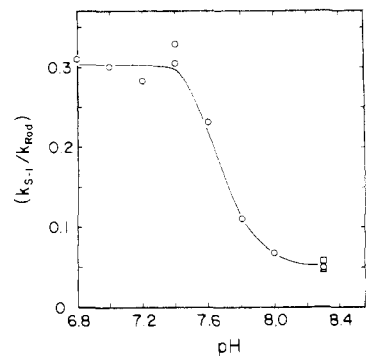

FIGURE 2: Titration curve of the "normalized" rate of cross-linking myosin heads $k_{S\text{-}1}°$ (= $k_{S\text{-}1}/k_{rod}$). Cross-linking was carried out in (a) 80 mM KCl, 40 mM imidazole, and 0.1 mM $MgPP_i$ (pH 6.8–7.4) or (b) 80 mM KCl and 40 mM triethanolamine (pH 7.4–8.3). Cross-linking was also carried out in solvents in the presence of 1 mM $CaCl_2$ (□) and 1 mM EGTA (△) at pH 8.3. The upper point at pH 7.4 was obtained using Tris buffer and the lower point using imidazole buffer.

at each pH and digested with chymotrypsin in the presence of EDTA (1 mM) to cleave the head–rod linkage (see Weeds & Taylor, 1975; Bagshaw, 1977). The time-dependent loss of non-cross-linked S-1 and rod was derived from densitometry of $NaDodSO_4$ gels following electrophoresis as described in Materials and Methods. Figure 1 presents a typical plot of log relative intensity vs. time of cross-linking each fragment, showing that the apparent first-order rate constant of cross-linking the rod segment (k_{rod}) is about nine times that of the head segment ($k_{S\text{-}1}$) at pH 7.8. The rate of the amidination reaction between lysine side chains and imidate esters of DMS varies with pH (Hunter & Ludwig, 1962). Thus, $k_{S\text{-}1}$ 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 within the thick filament, the ratio of the two rate constants, $k_{S\text{-}1}$ and k_{rod}, was compared at each pH. The ratio $k_{S\text{-}1}/k_{rod}$ (= $k_{S\text{-}1}°$) should change only if the effective contact area between myosin heads and the thick filament surface varies with pH since k_{rod} and $k_{S\text{-}1}$ are expected to show the same pH dependence of the amidination reaction. Moreover, k_{rod} should be much less sensitive to a change in the surface arrangement of the heads than $k_{S\text{-}1}$ due to the fact that a large portion of the rod segment is locked within the thick filament core. Thus, the normalized rate constant of cross-linking myosin heads, $k_{S\text{-}1}°$, can be used to estimate how easily myosin heads are cross-linked to the filament core.

The titration curve, $k_{S\text{-}1}°$ vs. pH, is presented in Figure 2. On elevating the pH from 6.8 to 7.4, $k_{S\text{-}1}°$ remains unchanged at a value of ~0.3, while it shows a sharp decrease over a narrow pH range (pH 7.4–8.0) leveling off at a value of ~0.04 above pH 8.0.

The presence of 1 mM $CaCl_2$ or 1 mM EGTA in the cross-linking solvent had no effect on $k_{S\text{-}1}°$ at pH 8.3.

Ultracentrifugation of Synthetic Thick Filaments. The decreased rate of cross-linking myosin heads on elevating the pH does not result from a pH-dependent dissociation of the thick filament into monomeric myosin. Before the cross-linking reaction, synthetic thick filaments in 80 mM KCl and 40 mM triethanolamine (pH 8.3) showed a hypersharp polymer peak in the ultracentrifuge with no detectable monomer under these ionic conditions in agreement with the observations of Josephs & Harrington (1966). After 6-h cross-linking, 2 M KCl was added to an aliquot of the filament solution to give a final KCl concentration of 0.5 M. Only a single, slightly broadened sedimenting boundary corresponding to the synthetic filament was observed in the high ionic strength solvent demonstrating that the filament is completely cross-linked and, therefore, not dissociable under these conditions.

Cross-linking of Synthetic Thick Filaments Following Preincubation with a Lysine-Specific Monofunctional Reagent. At neutral pH, myosin heads are cross-linked to the filament surface with a rate about one-third that of the rod–rod cross-linking reaction (see Figure 2). This finding suggests that a fairly large number of ε-amino groups located within the myosin heads take part in the cross-linking reaction, since a large number of lysine side chains are likely to be participating in the cross-linking reaction of the rod segments due to the close packing of these segments in the filament core. Alternatively, it is possible that a few lysine side chains with high reactivity toward imidate esters reside in positions favorable for the head–surface cross-linking reaction and that the effective contact surface area between the head and the filament surface is small. In the latter case, a pH-dependent shift in the local environment of these ε-amino groups might change their reactivity and consequently the rate of cross-linking myosin heads without any change in the effective contact area between the cross-bridge and the thick filament surface.

To answer this question, we first modified the synthetic thick filament with a monofunctional imidate ester, methyl acetimidate (molar ratio of myosin to methyl acetimidate, 1:500) for 2 h at pH 7.2. As a result of the methyl acetimidate treatment, any highly reactive lysine side chains should be rapidly blocked. Following treatment with the monofunctional reagent, titration of free lysine side chains with trinitrobenzenesulfonic acid showed that about 10 residues of the total lysine side chains of myosin were modified under these conditions (see Materials and Methods).

Synthetic thick filaments with and without methyl acetimidate modification were then cross-linked with DMS under the same solvent conditions and the time courses of cross-linking myosin heads and that of rod segments monitored. The kinetics of methyl acetimidate modified and nonmodified synthetic thick filaments is shown in Figure 3, where it can be seen that the rate of cross-linking myosin heads as well as that of rod segments remains unchanged following preincubation with methyl acetimidate. Thus, it seems likely that the cross-linking reaction of both myosin heads and rod segments involves a large number of lysine side chains of comparable

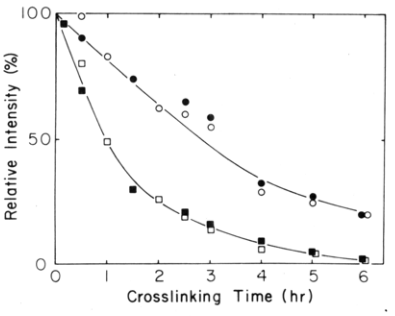

FIGURE 3: Time course of cross-linking the head (○, ●) and rod, (□,■) segments in synthetic thick filaments after prior modification with a monofunctional reagent (methyl acetimidate). Open symbols: nonmodified filament. Closed symbols: methyl acetimidate modified filament. Cross-linking conditions: solvent, 80 mM KCl, 40 mM imidazole, and 0.1 mM $MgPP_i$ (pH 7.2); myosin filament, 1 mg/mL; cross-linker, dimethyl suberimidate (1.5 mg/mL); temperature, 4 °C.

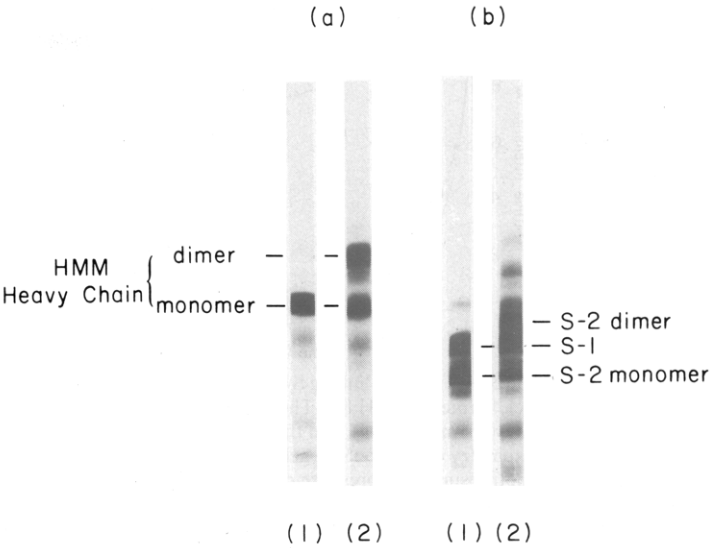

FIGURE 4: (a) Acrylamide–agarose/$NaDodSO_4$ gels of HMM (1) before and (2) after cross-linking. Cross-linking conditions: solvent, 80 mM KCl and 40 mM imidazole (pH 7.4); HMM, 1 mg/mL; cross-linker, dimethyl suberimidate, 1.2 mg/mL; cross-linking time, 2 h; temperature, 4 °C. (b) Acrylamide–agarose/$NaDodSO_4$ gels of chymotryptic product of HMM (1) without and (2) with cross-linking. Cross-linking conditions are the same as in a. Conditions for the chymotryptic digestion: solvent, 0.1 M KCl, 20 mM phosphate, and 2 mM EDTA (pH 6.9); HMM, 1 mg/mL; chymotrypsin, 0.02 mg/mL; digestion time, 15 min; temperature, 25 °C. These gels were overloaded to detect cross-linked products.

chemical reactivities rather than a small number of highly reactive lysine side chains.

Cross-linking of HMM. In an earlier paper (Sutoh & Harrington, 1977) we argued from indirect evidence that the rapid cross-linking of myosin heads within the thick filament observed at neutral pH results from formation of intermolecular covalent bridges with the filament surface and not from formation of intramolecular bridges between the head and rod or between the two heads of an individual molecule. We now wish to provide direct evidence in support of this conclusion from cross-linking studies of HMM. It is important to establish this point unambiguously since the decrease in the rate of cross-linking myosin heads observed on elevating the pH could be interpreted as resulting from a structural change within an individual molecule rather than from a change in the effective contact area between the head and the thick filament surface.

HMM was reacted with DMS under conditions in which 50% of the myosin heads would be cross-linked in the synthetic thick filament. As shown in Figure 4a, about 60% of the HMM heavy chain (150 000) is cross-linked to form the heavy chain dimer (300 000), but there is no indication of intermolecular cross-linking between HMM particles to form a species larger than 300 000. Cross-linked and non-cross-linked HMM were then digested with chymotrypsin in the presence of EDTA. The major products of the chymotryptic digestion (Figure 4b) are S-1 (90 000) and S-2 (60 000) per single chain (see Materials and Methods). Densitometer traces of the HMM, S-1, and S-2 bands in these $NaDodSO_4$ gels showed that ~95% of the HMM heavy chain was digested to S-1 and S-2 without further internal cleavage within particles. Following the cross-linking reaction with DMS, the chymotryptic product exhibits an additional intense band just above the S-1 band together with several faint bands and there is a concomitant decrease in the intensity of the S-2 band. When the cross-linking reaction was carried out after digesting HMM to S-1 and S-2, the new band again develops rapidly above the S-1 band.

Taking into account the apparent molecular weight of the new band (120 000), the above findings show that this band corresponds to a cross-linked dimer of the S-2 peptide chains. From densitometry traces of each band (Figure 4b) it is clear that the intensity of the S-1 band remains virtually unchanged after the cross-linking reaction, consistent with the observation that only a trace amount of material is observed in the region above the S-2 dimer in the gel in Figure 4b (cross-linked S-1 dimer and cross-linked S-1–S-2 dimer are expected to appear above the S-2 dimer). Thus cross-linking of the S-1 segment to its own S-2 segment as well as cross-linking between the two heads of an individual myosin molecule are extremely slow reactions compared with the reaction which is responsible for cross-linking myosin heads to the thick filament surface.

The presence of 5 mM MgATP, 1 mM MgADP, and/or 1 mM $CaCl_2$ during the HMM cross-linking reactions does not alter the gel pattern shown in Figure 4b.

Kinetics of Cross-linking HMM Segments within the Thick Filament. In view of the results presented above, it seems likely that myosin heads are released from the filament surface at alkaline pH and it was therefore of interest to determine if only the head segment is released or whether the entire HMM segment swings away from the surface. To answer this question we measured the rate of cross-linking the HMM heavy chain segment in synthetic filaments at pH 8.3. Synthetic thick filaments at various stages of cross-linking were digested with chymotrypsin in the presence of Ca ions (1 mM). Under these conditions the protease-sensitive site at the head–rod linkage remains intact, whereas the LMM–HMM site is rapidly cleaved to form the HMM fragment (Bagshaw, 1977; Weeds & Pope, 1977; see Materials and Methods).

$NaDodSO_4$ gels of the proteolytic products showed the presence of an HMM heavy chain band (150 000) and a LMM band (60 000) together with a myosin heavy chain band. Plots of the relative intensity of the HMM and LMM bands vs. time of cross-linking (Figure 5) reveal that the rate of cross-linking HMM to the thick filament surface at pH 8.3 approximates that of LMM. Moreover, HMM heavy chain dimer was not detected in significant amounts on the $NaDodSO_4$ gels throughout the cross-linking reaction, probably due to the cross-linking of the S-2 moiety to the thick filament surface. Thus, assuming that the lysine residues are distributed at random over the rod surface, it appears that a significant portion of the chymotryptic S-2 segment resides close to the thick filament surface even when the myosin heads move out from the surface at alkaline pH.

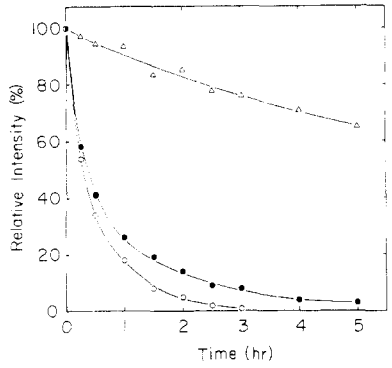

FIGURE 5: Time course of cross-linking S-1, HMM, and LMM segments in synthetic thick filaments at pH 8.3. The relative intensities of the S-1 (△), HMM heavy chain band (●), and the LMM band (○) were determined by densitometry of $NaDodSO_4$ gels of cross-linked filaments digested by chymotrypsin in the presence of 1 mM $CaCl_2$. Cross-linking conditions: solvent, 80 mM KCl and 40 mM triethanolamine (pH 8.3); myosin filament, 1 mg/mL; dimethyl suberimidate, 0.6 mg/mL; temperature, 4 °C.

This interpretation is consistent with the pH dependence of cross-linking the rod in synthetic thick filaments described earlier. Unlike the S-1 subunit, the rate of this reaction increases gradually over the pH range 6.8–8.3. At pH 8.3 the first-order rate constant is about twice that at pH 6.8 in accord with the pH dependence expected for the amidination reaction. This finding is contrary to the result expected if the long S-2 segment, which makes up about 50% of the rod, hinges away from the thick filament surface at high pH.

Discussion

The decrease in the normalized rate constant ($k_{S\text{-}1}°$) of cross-linking myosin heads in synthetic thick filaments observed in the present study over the pH range 7.4–8.0 probably originates from a change in the arrangement of myosin heads along the filament surface. Dissociation of the filament during the cross-linking reaction cannot account for the pH-dependent transition since the sedimentation boundary of the filament remains unaltered and no monomer peak is observed in 0.5 M KCl after the cross-linking reaction. Nor can we ascribe the $k_{S\text{-}1}°$ vs. pH profile to a shift in intramolecular cross-linking between the head and its own rod segment in view of the finding that S-1 is not cross-linked to S-2 in isolated HMM. It is also unlikely that changes in the effective concentration of a small number of highly reactive lysine side chains could be responsible for the pH-dependent phenomenon since preincubation with a monofunctional imidate ester has no effect on the subsequent time course of cross-linking. The simplest interpretation of the present results is that the myosin heads in the thick filament take two different positions with respect to the filament surface depending on pH; at neutral pH all of the myosin heads are in close contact with the filament surface and are therefore rapidly cross-linked to the filament core, whereas above pH 8 they move away from the filament surface and can be cross-linked to the surface only during occasional thermal collisions. This interpretation is supported by fluorescence depolarization experiments. At neutral pH the Brownian motion of fluorophore-labeled myosin heads in synthetic thick filaments is highly restricted, whereas at pH 8.3 the mobility of the heads approaches that of dispersed myosin molecules (Mendelson & Cheung, 1976).

An alternative explanation for our cross-linking observation is that some type of conformational change is induced in the head moiety on shifting the pH, resulting in a drastic alteration in the distribution of ϵ-amino groups available for the cross-linking reaction, though it seems to us unlikely that such a large conformational change occurs simply by raising the pH from 7.4 to 8.0. The relatively sharp pH dependence may be due to titration of ionizing side groups over this range. The resulting increase in net charge on the protein could lead to electrostatic repulsion between the S-1 subunit and the filament surface. According to Mihalyi (1950), about 75 histidine residues in the myosin molecule (~40 are confined to the S-1 subunits) are titrated over the pH range 6.3–8.2 based on the apparent heat of dissociation of the ionizing groups.

In earlier studies (Sutoh & Harrington, 1977) the time course of cross-linking myosin heads (with dimethyl dithiobispropionimidate (DTBP) or methyl mercaptobutyrimidate) in synthetic thick filaments at neutral pH and physiological ionic strength was determined in the presence of various ligands including MgATP, MgADP, magnesium pyrophosphate, and/or $CaCl_2$. DTBP was also employed to cross-link glycerinated myofibrils in rigor at rest length. The (normalized) time courses of cross-linking myosin heads to the thick filament surface under all of these conditions were indistinguishable suggesting that neither the presence of the ligands nor the shift from resting to rigor state (at rest length) results in release of myosin heads from the filaments surface. In resting muscle, the surface of the actin filament is at a radius of 180–190 Å from the center of the thick filament (Huxley & Brown, 1967). If we assume a three- or four-stranded model for the thick filament (Squire, 1972), consistent with recent biochemical studies (Tregear & Squire, 1973; Morimoto & Harrington, 1974), the center of mass of myosin projections would extend to a radius of 150–200 Å (Squire, 1972) and therefore may be of sufficient length to span the gap between thick and thin filament surfaces. When muscle contracts the double hexagonal lattice of filaments within the A-band region of the sarcomere expands and the gap between the thick and thin filament surfaces increases by as much as 50 Å (Elliott et al., 1963). Thus to allow interaction with actin at all sarcomere lengths during contraction would seem to require movement of the heads from the thick filament surface. To accommodate the changing lateral distance between filaments, it has been proposed (Huxley, 1969) that the protease-sensitive site between the LMM and HMM segments of myosin might act as a hinge, allowing the S-2 segment of HMM to swing freely away from the thick filament core.

The present cross-linking experiments, when taken in conjunction with the earlier studies at neutral pH suggest, however, that a significant fraction of the S-2 segment is held close to the filament surface under all solvent conditions which we have examined to date including high pH where the heads move away from the thick filament backbone. Though we do not rule out the possibility that the whole HMM segment swings away from the thick filament surface during a contractile cycle through the opening of the protease-sensitive LMM-HMM "hinge" region, it seems possible that release of the myosin head at short sarcomere lengths is a localized phenomenon involving only the head segment and possibly a part of the S-2 region, as in the case of the pH-dependent shift of the head observed in the present study.

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Conformational States of a Hydrophobic Protein. The Coat Protein of fd Bacteriophage†

Yasuhiko Nozaki, Jacqueline A. Reynolds, and Charles Tanford*

ABSTRACT: The coat protein of fd bacteriophage has a short polypeptide chain of only 50 amino acid residues, containing a highly hydrophobic segment of 19 amino acids that is entirely devoid of ionic or other strongly polar amino acids. In the viral particle the protein exists as a closely packed array of α helices. It can be transformed to a monomeric randomly coiled polypeptide in very concentrated ($\geq$7.3 M) guanidinium chloride. In anionic detergents or phospholipids the protein is dimeric, with a mixed conformation ("50% α"), the hydrophobic segment having a β structure, whereas the two ends are predominantly α helical. In guanidinium chloride at concentrations of 6 M or less, and under other conditions in the absence of an anionic detergent or phospholipid, the protein forms an intractable polymer, with a β-type conformation. If the protein is succinylated an oligomeric form of this structure (speculatively thought to be a soluble variety of a "β barrel") can be obtained as a metastable state. The 50% α conformation, the β oligomer, and the random coil can be interconverted reversibly, but formation of the β polymer appears to be irreversible.

The coat protein of fd bacteriophage has a single polypeptide chain of 50 amino acid residues, with a remarkable amino acid sequence (Nakashima & Konigsberg, 1974), shown in Figure 1. The polypeptide contains an uninterrupted sequence of 19 uncharged and predominantly hydrophobic residues (positions 21–39), 12 of them having side chains that can gain between 1300 and 3400 cal/mol apiece if they can be removed from contact with water (Nozaki & Tanford, 1971). The threonine residue at position 36 is the only one in this sequence that can be considered significantly hydrophilic. This segment of polypeptide chain must have an exceptionally strong propensity to form structures in which contact with an aqueous medium is avoided, and the behavior of the coat protein in various solvent systems might therefore be expected to be anomalous when compared to water-soluble polypeptides with a more even distribution of hydrophilic and hydrophobic residues along the chain.

The only other known polypeptide chain with a comparable amino acid sequence is that of the transmembrane MN-glycoprotein of the human erythrocyte (Tomita & Marchesi, 1975). It has a hydrophobic segment that is 23 residues long, but there are three hydrophilic residues within it, two threonines and one serine. Another membrane-bound protein, microsomal cytochrome b_5, has two eight-residue hydrophobic segments in its membrane-embedded region, but they are separated from each other by 13 intervening residues (Corcoran & Strittmatter, 1977). Sequence data for other intrinsic membrane proteins are not available, but it is considered likely that many of them may contain polypeptide segments of this type. Studies of the fd coat protein may therefore be of general interest in relation to the solution behavior of membrane proteins. The fd virus itself does not possess a membrane and the coat protein in the virus is not associated with lipid or any other amphiphilic substance. The protein does, however, reside in the phospholipid membrane of its host bacterium, *Escherichia coli,* during the process of viral biosynthesis and assembly (Smilowitz et al., 1972; Webster & Cashman, 1973); i.e., it is capable of existing as a membrane protein.

Previous work has established that the coat protein exists in the native virus as a closely packed layer of helices, with essentially all of the polypeptide backbone in an α-helical conformation (Marvin et al., 1974; Marvin & Wachtel, 1975; Nozaki et al., 1976). The virus may be disrupted by use of deoxycholate, and the purified protein in this detergent has a quite different conformation, judged by circular dichroism measurements to contain about 50% α helix and about 30% β

†From the Whitehead Medical Research Institute and the Department of Biochemistry, Duke University Medical Center, Durham, North Carolina 27710. *Received October 4, 1977.* This work was supported by Research Grants AM-04576 from the National Institutes of Health and PCM7615240 from the National Science Foundation. Charles Tanford is a Research Career Awardee of the National Institutes of Health.

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