Cross-Linking of Myosin Thick Filaments under Activating and Rigor Conditions. A Study of the Radial Disposition of Cross-Bridges†

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ABSTRACT: The bifunctional cross-linking reagents, dimethyl 3,3′-dithiobispropionimidate (DTBP) and methyl 4-mercaptobutyrimidate (IM-SH), were employed to detect changes in the radial disposition of myosin heads induced by the presence of Ca ions or by the shift from resting to rigor state. Synthetic thick filaments were cross-linked with DTBP or IM-SH/H_2O_2 in a solvent consisting of 80 mM KCl, 40 mM imidazole, and 5 mM MgATP (pH 7.4 or 6.8) in the presence and absence of Ca ions. The cross-linking reaction was also carried out in the presence of MgADP and $MgPP_i$. The fraction of myosin heads cross-linked to the thick filament surface at various stages of reaction was determined by the extent of cleavage of head segments (subfragment 1) from the thick filament aggregate by papain or chymotrypsin. Both cross-linkers bridge head segments as well as rod segments of myosin very effectively under all solvent conditions examined, suggesting that the myosin heads are always in close contact with the filament surface. Furthermore, formation of cross-links between two heads of the same myosin molecule in synthetic thick filaments was not detected during the cross-linking reaction. Since the time course of cross-linking myosin heads to the thick filament surface remains invariant with changes in the solvent environment, it is unlikely that the cross-bridges swing away from the thick filament surface on binding Ca ions. DTBP has also been employed to cross-link glycerinated myofibrils in rigor at rest length. Myosin heads in the rigor myofibril are cross-linked to the thick filament surface much more rapidly than to the thin filament despite the fact that a major fraction of heads are bound to the thin filament. The time course of cross-linking myosin heads to the thick filament surface in the rigor myofibril is indistinguishable from that observed in the synthetic thick filaments, suggesting that the radial disposition of the heads in both systems is closely similar, if not identical. We conclude from these results that neither the presence of Ca ions nor the shift from resting to rigor state (at rest length) results in release of myosin heads from the thick filament surface.

Haselgrove (1970, 1975) and Huxley (1972) have observed that activation of muscle is accompanied by a substantial change in the layer line intensities of x-ray reflections from cross-bridges, suggesting considerable cross-bridge movement, even when muscles are stretched to a sarcomere length where interaction of actin and myosin filaments should be abolished. Although other explanations can be advanced to account for this finding (Huxley, 1972), one intriguing possibility is that some type of Ca-activated cross-bridge release mechanism exists within the thick filament. Such a Ca-activating switch might act to unlock the highly ordered resting state orientation in which cross-bridges are rigidly held in the proximity of the thick filament backbone (Huxley and Brown, 1967), and allow them to move into the vicinity of the thin filament surface. Consistent with this idea, it has recently been shown (Werber et al., 1972; Morimoto and Harrington, 1974b) that the 5,5′-dithiobis(2-nitrobenzoic acid) (Nbs_2)[1] light chain in each myosin head binds one Ca ion very tightly (with binding constant 1.7×10^5 M^{-1} at a free Mg ion concentration of 0.3 mM). Moreover, the sedimentation and viscosity properties of native as well as synthetic thick filaments undergo a detectable and reversible change on addition of Ca ions with midpoint of the transition of pCa = 5.2 (Morimoto and Harrington, 1974b). Although these changes have been attributed to some type of Ca-dependent conformational transition within the thick filament, possibly to a shift in the radial disposition of myosin heads, Nihei et al. (1974) and, more recently, Mendelson and Cheung (1976) have reported experiments which appear to contradict this interpretation. Nihei et al. (1974) have investigated the orientation of cross-bridges in glycerinated muscle fibers using fluorescence polarization. When such muscle fibers are stretched to a length which eliminates overlap between thin and thick filaments, the resting-state orientation of cross-bridges is unaffected by immersion in either rigor-inducing or contraction-inducing solutions. Mendelson and Cheung (1976) have observed that the rotational Brownian motion of fluorophore-labeled myosin heads in synthetic thick filaments is unaffected by Ca ions, suggesting that the myosin heads do not increase their rotational mobility on binding this divalent ligand, as would be expected if they were released from the thick filament surface.

In seeking to resolve this dilemma, it should be noted that the x-ray diffraction patterns can be interpreted as indicating that the outer tip of the cross-bridge (resting state) is situated at a position quite close to its neighboring thin filament surface. Squire (1972) proposed that the thick filament could well be three or four stranded rather than two stranded as originally proposed (Huxley and Brown, 1967) and recent biochemical studies (Tregear and Squire, 1973; Morimoto and Harrington, 1974a) are more consistent with these models. In either case,

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[1] Abbreviations used are: Nbs_2, 5,5′-dithiobis(2-nitrobenzoic acid); DTBP, dimethyl 3,3′-dithiobispropionimidate; IM-SH, methyl 4-mercaptobutyrimidate; $PhMeSO_2F$, phenylmethylsulfonyl fluoride; Temed, *N,N,N′,N′*-tetramethylenediamine; S-1, subfragment 1; OD, optical density; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N′*-tetraacetic acid; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane.

the myosin cross-bridges in a relaxed muscle would be expected to have a radial projection of 180–200 Å compared to ~130 Å in the two-stranded model. Calculating the expected equatorial x-ray reflections based on the Squire "long arm" model, Lymn and Cohen (1976) have recently shown that the change in the characteristic ratio ($I_{1,1}/I_{1,0}$) of the equatorial x-ray reflections, observed when vertebrate striated muscle is activated or passes into rigor, can be simulated on the assumption that the cross-bridges simply change their angle or azimuthal tilt without significant radial movement. Although this interpretation has been challenged (Haselgrove et al., 1976), it raises the tantalizing possibility that the lower surface of the cross-bridges might never leave the vicinity of the thick filament when muscle contracts or passes into rigor.

In the present study, we have attempted to obtain some clarification of the questions mentioned above by investigating the kinetics of cross-linking of myosin heads to the thick filament surface under a variety of solvent conditions expecting that the rate of the cross-linking reaction should be a sensitive indicator of changes in the radial disposition of myosin heads within the thick filament. We employed two imido ester cross-linkers, dimethyl 3,3′-dithiobispropionimidate (DTBP, Wang and Richards, 1974) and methyl 4-mercaptobutyrimidate (IM-SH, Traut et al., 1973). These reagents react very specifically with lysine side chains, and the resulting amidinated proteins are expected to maintain their chemical and physical properties since their charge balance remains unchanged by the chemical reaction (Hunter and Ludwig, 1962).

Experiments with synthetic thick filaments under various solvent conditions and glycerinated myofibrils in rigor are described. They demonstrate that the proximal surfaces of myosin heads in the synthetic thick filament are in contact with the thick filament surface both in the presence and absence of Ca ions. Moreover, assuming that the filament lattice remains invariant during the cross-linking reaction, it appears that myosin heads span the gap between thin and thick filaments in the rigor myofibril at rest length.

Materials and Methods

Reagents and Proteins. Dimethyl 3,3′-dithiobispropionimidate, methyl 4-mercaptobutyrimidate, and dimethyl malonimidate were purchased from Pierce; dithiothreitol, ATP, ADP, catalase (crystalline), and phenylmethylsulfonyl fluoride ($PhMeSO_2F$) were from Sigma; 2-mercaptoethanol, 2-iodoacetamide, acrylamide, *N,N′*-methylenebisacrylamide, and *N,N,N′,N′*-tetramethylethylenediamine (Temed) were from Eastman Kodak; chymotrypsin and papain were from Worthington Biochemical; H_2O_2 was from Baker. All other chemicals were reagent grade.

Preparation of rabbit skeletal myosin is described elsewhere (Godfrey and Harrington, 1970a). Actin was prepared according to Bailin and Bárány (1972). The native thin filament preparation was kindly supplied by Dr. J. Godfrey in this laboratory and the method of its preparation will appear elsewhere. Subfragment 1 (S-1) and myosin rod were prepared according to Lowey et al. (1969). Myofibrils from rabbit psoas muscle were prepared according to Kundrat and Pepe (1971).

Myosin concentration was determined spectrophotometrically employing $E_{280}^{1\%} = 5.5$ (Godfrey and Harrington, 1970a). Actin concentration was determined according to Lymn and Taylor (1971). The approximate concentration of myofibrils was determined by dissolving them in 2% sodium dodecyl sulfate and measuring OD_{280}, assuming $E_{280}^{1\%} \simeq 7$.

Cross-Linking of Synthetic Thick Filaments by DTBP; Proteolytic Digestion of the Cross-Linked Filament. All cross-linking and digestion procedures were carried out at 4 °C. Synthetic thick filaments (1 mg/mL) were formed at pH 8.3 according to Josephs and Harrington (1966). The filament solution was first dialyzed against solvent consisting of 80 mM KCl and 40 mM imidazole (pH 7.4), and to the dialyzed filament solution was added 0.1 vol of ligand solution (50 mM MgATP, 10 mM MgADP, 1 mM magnesium pyrophosphate ($MgPP_i$), 10 mM $CaCl_2$, and/or 10 mM EGTA) which had been preadjusted to pH 7.4. The cross-linking reaction was started by addition of DTBP (in 80 mM KCl and 40 mM imidazole, pH 7.4).

Two procedures were used for cross-linking: (1) a constant concentration of cross-linker (0.6 mg/mL) with varying times of cross-linking (0–6 h); (2) a constant cross-linking time (16 h) with varying concentrations of cross-linker (0–0.5 mg/mL). The decay curves of the relative intensity of the S-1 bands on sodium dodecyl sulfate gels of cross-linked and digested synthetic thick filaments (see Figure 2) obtained by procedures 1 and 2 are superimposable when the abscissa scales are normalized using the intensity change of the heavy chain band as described later. Thus, it appears that the heads are cross-linked to equivalent levels by the two procedures.

The cross-linking reaction was quenched by addition of 0.2 vol of 2.5 M ethanolamine (pH 8.0). When partially cross-linked filaments were digested with enzymes to cleave the myosin heads from the rod segment (see below), the cross-linking reaction was quenched only after proteolysis since high concentrations of ethanolamine change the specificity of proteolytic digestion.

Proteolysis of filaments at various stages of cross-linking was carried out by addition of 0.1 vol of papain (0.2 mg/mL in 5 mM cysteine, 1 mM EDTA, and 0.1 mM dithiothreitol, pH 6.0). Digestion was allowed to proceed for 5 min and terminated by addition of 0.1 vol of 0.5 M 2-iodoacetamide. In some experiments chymotrypsin was used in place of papain. In this case, 0.1 vol of 0.1 M EDTA (pH 7.0) was added first, followed by 0.1 vol of 3 mg/mL chymotrypsin, since this enzyme digests only the junction between rod and head segments of myosin under these conditions (Balint et al., 1971; Weeds and Taylor, 1975). Digestion was allowed to proceed for 10 min and then terminated by addition of 0.1 vol of 10 mg/mL $PhMeSO_2F$ in 2-propanol (Farney and Gold, 1963). After the cross-linking and proteolysis steps and before denaturing the modified protein in sodium dodecyl sulfate, either catalase or cytochrome *c* was added as an internal standard. This procedure was adopted in all of our cross-linking studies.

Cross-Linking of Synthetic Thick Filaments by IM-SH; Proteolytic Digestion of the Cross-Linked Filaments. All cross-linking and digestion procedures were carried out at 4 °C. Synthetic thick filaments (1 mg/mL) were formed in 0.1 M KCl and 20 mM triethanolamine (pH 8.3). The synthetic thick filaments were first amidinated by addition of 0.05 vol of 2 mg/mL IM-SH in 0.1 M KCl, 20 mM triethanolamine, and 0.1 M dithiothreitol (pH 8.3). This reaction was allowed to proceed for 6 h, and the amidinated filaments were then dialyzed against solvent consisting of 80 mM KCl and 40 mM imidazole (pH 6.8 or 7.4).

After adding various ligands to the dialyzed filament solution as before, the second step of the cross-linking reaction was initiated by addition of H_2O_2 to a final concentration of 10 mM. The oxidation of sulfhydryl groups by H_2O_2 was allowed

to proceed for various periods of time (up to 6 h) and this reaction was quenched by addition of 0.03 vol of 1 mg/mL catalase. Following this step, the free sulfhydryl groups were blocked by addition of 0.1 vol of 0.5 M 2-iodoacetamide. When the cross-linked filaments were digested (see above) by a proteolytic enzyme, 2-iodoacetamide was added after the digestion.

Cross-Linking of Glycerinated Myofibrils: Proteolytic Digestion of the Cross-Linked Myofibrils. All cross-linking and digestion procedures were carried out at 4 °C. Glycerinated myofibrils (3 mg/mL) suspended in 80 mM KCl, 40 mM imidazole, and 1 mM EGTA (pH 7.4) were cross-linked by DTBP for 16 h, varying the cross-linker concentration up to 0.5 mg/mL. The cross-linking reaction was quenched by addition of 0.2 vol of 2.5 M ethanolamine (pH 8.0). When the cross-linked myofibrils were digested by a proteolytic enzyme, the cross-linking reaction was quenched after the digestion. Digestion was carried out with 0.1 mg/mL papain for 5 min or 0.2 mg/mL chymotrypsin for 10 min and then terminated. When chymotrypsin was used, 0.1 vol of 0.1 M EDTA (pH 7.0) was added prior to addition of the enzyme.

Modification of Glycerinated Myofibrils with Dimethyl Malonimidate (DMM). All procedures were carried out at 4 °C. Glycerinated myofibrils (3 mg/mL) in 80 mM KCl, 40 mM imidazole, and 1 mM EGTA (pH 7.4) were treated with dimethyl malonimidate (2.5 mg/mL) for 4 h. The reaction was terminated with ethanolamine. Chymotryptic digestion of modified myofibrils was carried out as described above.

Sodium Dodecyl Sulfate Gel Electrophoresis. Sodium dodecyl sulfate gel electrophoresis was carried out according to Weber and Osborn (1969), employing the Tris–buffer system. In this study, we routinely used 5% gels (acrylamide, 50 mg/mL; methylenebisacrylamide, 1.5 mg/mL). When it was necessary to detect species of high molecular weight, 2.5% acrylamide–0.5% agarose gels were used (Reisler et al., 1973). This system allows penetration of cross-linked species with molecular weights up to 2×10^6.

Protein samples were first denatured in 2% sodium dodecyl sulfate and 50 mM Tris (pH 8.0) at 37 °C for 16 h, and then dialyzed against 0.1% sodium dodecyl sulfate and 10 mM Tris (pH 8.0). In order to cleave the interior disulfide bonds of the cross-links, cross-linked protein samples were dialyzed against 0.1% sodium dodecyl sulfate, 10 mM Tris, and 2% 2-mercaptoethanol (pH 8.0) for 1 week at room temperature. Gel loadings were adjusted to ensure that each band after electrophoresis contained less than 10 μg of material. The bands were identified by their position relative to purified myosin, rod, and S-1, which were co-electrophoresed with the samples.

To determine the extent of cross-linking of myosin heavy chain, the proteolytic fragments of myosin, or other muscle proteins after the cross-linking reaction, the following procedures were adopted.

Densitometer recordings of sodium dodecyl sulfate gels were traced on heavy weight paper and each peak cut out and weighed. All peak areas were normalized using the peak area of the standard protein (cytochrome *c* or catalase) 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 such cross-linked samples was expressed as "relative intensity (%)," setting the relative intensity of the corresponding band on the corresponding standard gel as 100% (see Figure 2). Thus the relative intensity of any migrating band converges to 100% at zero cross-linking time (or zero cross-linker concentration) regardless of its absolute intensity.

Method for Compensating Differences in the Overall Rate of Cross-Linking Reactions. In order to compare the kinetics obtained with the two cross-linking reagents under various conditions, it is necessary to normalize the data to compensate for differences in the overall rate of cross-linking. For this purpose we used the rate of cross-linking myosin heavy chains as a standard. We expected that the rate of cross-linking myosin heavy chains would be affected only slightly even when myosin heads change their position since the contribution resulting from the head–rod linkage to the rate of cross-linking myosin heavy chains would be ~30% (the rate of the intensity change in the S-1 band on sodium dodecyl sulfate gels is one-third that in the heavy chain band (Figure 2)). Empirically, the decay curve of the intensity of the heavy chain band always fits a single exponential; therefore, regardless of differences in cross-linking conditions, it is possible to make the relative intensities of heavy chain band at various levels of cross-linking fit a single decay curve simply by expanding (or reducing) the abscissa scales.

Myosin ATPase Activity. The CA-ATPase activity and the EDTA-ATPase activity were measured according to Kielley and Bradley (1956) and Kielley et al. (1956).

Results

General Features of the Cross-Linking Reaction of Synthetic Thick Filaments. Reisler et al. (1973) have reported that synthetic thick filaments can be readily cross-linked with dimethyl suberimidate to yield structures in which all myosin heavy chains are cross-linked. The resulting cross-linked filaments maintain their filamentous structure even in a high ionic strength solvent (0.5 M KCl). The formation of interfilament cross-links could be avoided in these studies by carefully controlling the conditions for the cross-linking reaction.

In the present study synthetic thick filaments were also readily cross-linked either with DTBP or IM-SH/H_2O_2. After allowing the reaction to proceed to a stage where almost all of the heavy chains were cross-linked to each other (as judged by sodium dodecyl sulfate gel electrophoresis), the resulting filaments were dispersed in a high ionic strength solvent (0.5 M KCl) and examined in the ultracentrifuge. All of the material sedimented as a single filament peak, though a slight broadening of the peak was observed compared to the non-cross-linked filament system in low ionic strength solvent (Josephs and Harrington, 1966). Thus, when synthetic thick filaments are subjected to the cross-linking reaction, they neither dissociate nor do they undergo significant interfilament cross-linking.

Figure 1a shows typical sodium dodecyl sulfate gels of synthetic thick filaments which were cross-linked by DTBP followed by chymotryptic digestion. Also shown is a sodium dodecyl sulfate gel of synthetic thick filaments digested with chymotrypsin in the absence of cross-linking. Three bands corresponding to myosin heavy chain, rod, and subfragment 1 (S-1) are seen on the gels as indicated. After the cross-linking reaction, these bands decrease in their intensities. At very early stages of cross-linking (not shown) cross-linked oligomeric heavy chains or rods were observed on gels of high porosity. These bands disappear in later stages of the reaction and only highly cross-linked material is observed at the top of the gels. No band corresponding to the dimer of S-1 was detected on these gels. This result clearly indicates that a myosin head is cross-linked to the thick filament surface much more rapidly than to the adjoining head in the same myosin molecule.

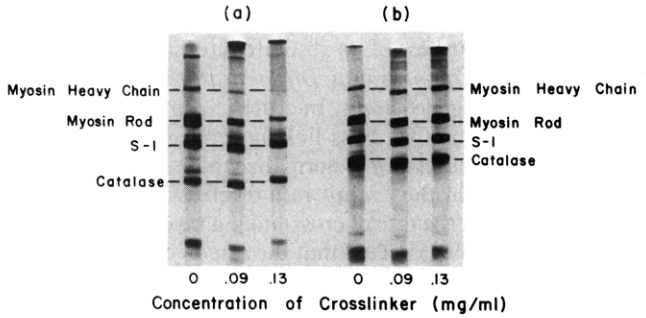

FIGURE 1: Sodium dodecyl sulfate gels (5% acrylamide) of chymotryptic digest of cross-linked synthetic thick filaments (a) before and (b) after cleavage of the cross-links by 2-mercaptoethanol. Solvent conditions for the cross-linking reaction: 80 mM KCl and 40 mM imidazole (pH 7.4); cross-linker, DTBP; cross-linking time, 16 h at 4 °C. The weak band above S-1 is a contaminant of disulfide-linked catalase dimer. The dimer is dissociated on reduction by 2-mercaptoethanol.

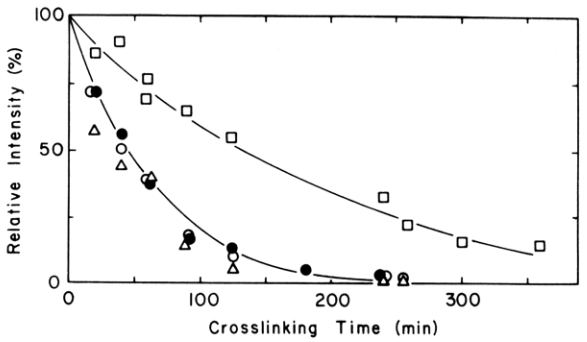

FIGURE 2: Time course of cross-linking various segments of myosin. The relative intensities of the heavy chain band (○), the rod band (△), and the S-1 band (□) are determined by densitometry of sodium dodecyl sulfate gels of cross-linked synthetic thick filaments digested with papain. The relative intensity of the heavy chain band of cross-linked synthetic thick filaments in the absence of proteolytic digestion is also shown (●). Solvent conditions for the cross-linking reaction: 80 mM KCl, 40 mM imidazole, and 0.1 mM $MgPP_i$ (pH 7.4); cross-linker, DTBP (0.6 mg/mL); temperature of cross-linking, 4 °C.

When the disulfide bond within each covalent bridge is cleaved by reduction in the presence of 2-mercaptoethanol, all the material can be recovered as monomeric segments (Figure 1b). The resulting mass distribution among the three bands corresponding to myosin heavy chain, rod, and S-1 is virtually identical for all gels regardless of the extent of cross-linking, demonstrating that the proteolytic digestion is unaltered by the cross-linking reaction.

The same result was obtained when the cross-links in synthetic thick filaments, which had been treated by IM-SH/H_2O_2 and then digested by papain, were cleaved by 2-mercaptoethanol.

Kinetics of Cross-Linking Various Segments of Myosin. Figure 2 shows the time-dependent changes in relative intensities (see Materials and Methods) of the heavy chain (○), rod (△), and S-1 (□) bands on sodium dodecyl sulfate gels when synthetic thick filaments were cross-linked with DTBP for varying times followed by papain digestion. Changes in the relative intensity of the heavy chain band at various stages of cross-linking of synthetic thick filaments in the absence of proteolytic digestion were also monitored (●). The change in the relative intensity of the heavy chain band before and after papain digestion follows the same decay curve showing again that the proteolytic digestion is apparently unaltered by the cross-linking reaction. The relative intensities of the heavy chain and rod bands decrease virtually at the same rate, consistent with the low degree of head–head cross-linking, whereas the rate of change in the relative intensity of the S-1 band is about one-third that of the heavy chain and rod bands. Since we expect the relative intensity of each band to be a measure of the relative fraction of myosin molecules in which a cross-link is formed in the corresponding segment, it appears that the rate of immobilizing the whole heavy chain or the rod segment of myosin is about three times as fast as the rate of immobilizing the head segment.

When synthetic thick filaments at various stages of cross-linking were digested with chymotrypsin in place of papain, the time course of cross-linking myosin heads was identical with that shown in Figure 2.

Effect of Interfilament Cross-Linking on the Time Course. As mentioned earlier, a small amount of interfilament cross-linking occurs during the reaction, although the protein concentrations employed strongly favor intrafilament cross-linking (Reisler et al., 1973). It was of interest to determine if such interfilament cross-links might affect the time course of cross-linking of myosin heads. We therefore followed the kinetics of cross-linking of the heads at a lower concentration of myosin (0.3 mg/mL). Cross-linking reactions were carried out in the presence of DTBP as the cross-linker. The resulting relative intensity vs. time plots were indistinguishable from those seen in Figure 2 showing that the change in the relative intensity of the S-1 band is independent of protein concentration. A similar result was obtained when IM-SH/H_2O_2 was used in place of DTBP.

Effect of Various Ligands on the Time Course of Cross-Linking Myosin Heads. To investigate the possibility that Ca ions can induce a change in the radial disposition of myosin heads in the synthetic thick filament, the cross-linking reactions were carried out in the presence (1 mM) and absence of this ligand. In this study, MgATP (5 mM) was also present in the solvent system (see Materials and Methods) since this divalent metal complex is an important physiological constituent of resting and contracting muscle. The ATP is gradually hydrolyzed by myosin over the course of the cross-linking reaction, but the cleavage rate is very slow in the presence of millimolar Mg ions and a major fraction of the nucleotide is present as MgATP at the end of the reaction. Figure 3 summarizes the results of this study. Within the limits of experimental error (±10% maximum), all points representing the relative intensity of the S-1 band after the cross-linking reaction fit a single decay curve regardless of the presence or absence of Ca ions. Similar studies were carried out in the presence of MgADP (1 mM) or $MgPP_i$ (0.1 mM) and the results are also included in Figure 3. None of these ligands induced a detectable change in the radial disposition of myosin heads in the synthetic thick filament. When IM-SH/H_2O_2 was used in place of DTBP as cross-linker, a similar result was obtained. Figure 4 shows the effect of various ligands on the time course of cross-linking myosin heads to the thick filament surface at pH 6.8 (the normalized decay curve remained unchanged when the MgATP–$CaCl_2$ and the MgATP–EGTA experiments were carried out at pH 7.4). If the two sets of data in Figures 3 and 4 are compared by normalizing the abscissa scales (see Materials and Methods), the change in the relative intensity of the S-1 band is found to fit a single decay curve.

General Feature of the Cross-Linking Reaction of Glycerinated Myofibrils in Rigor. In view of the results obtained in the previous section and the questions which were raised in the introductory statement, we have also investigated the radial disposition of myosin heads within the thick filaments of glycerinated myofibrils in rigor. Glycerinated myofibrils were first

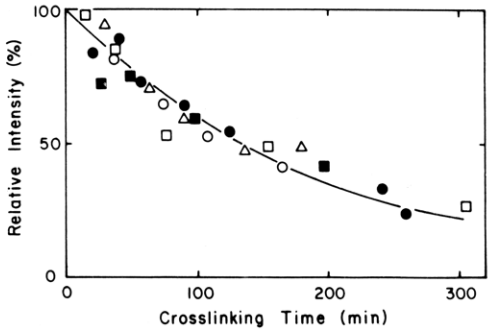

FIGURE 3: Effect of various ligands on time course of cross-linking myosin heads to the thick filament surface with DTBP: (△) no ligand; (●) 0.1 mM $MgPP_i$; (○) 1 mM MgADP; (□) 5 mM MgATP, 1 mM $CaCl_2$; (■) 5 mM MgATP, 1 mM EGTA. The buffer system consists of 80 mM KCl and 40 mM imidazole (pH 7.4). Temperature of cross-linking, 4 °C.

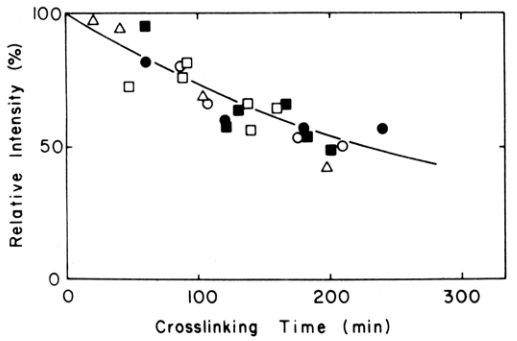

FIGURE 4: Effect of various ligands on time course of cross-linking myosin heads to the thick filament surface with IM-SH/H_2O_2. The buffer system consists of 80 mM KCl and 40 mM imidazole (pH 6.8). Temperature of cross-linking, 4 °C. All symbols in this figure have the same meaning as in Figure 3.

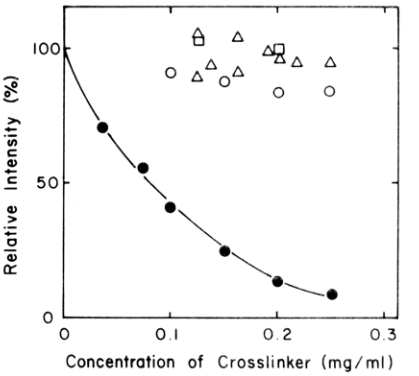

FIGURE 5: Comparison of the rate of cross-linking proteins of the thin filament with the rate of cross-linking myosin heavy chains in the rigor myofibril: (○) actin; (△) tropomyosin; (□) troponin-T; (●) myosin heavy chain. Solvent conditions for the cross-linking reaction: 80 mM KCl, 40 mM imidazole, and 1 mM EGTA (pH 7.4); cross-linker, DTBP; cross-linking time, 16 h at 4 °C.

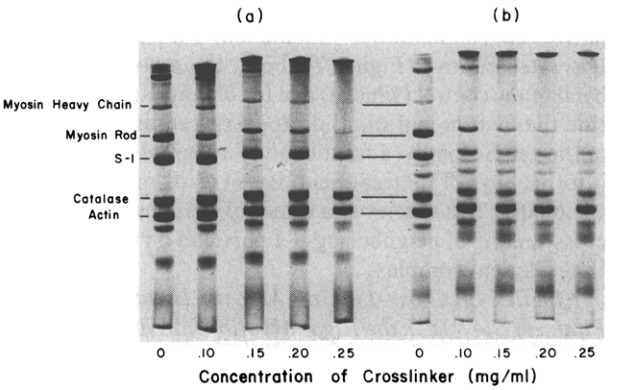

FIGURE 6: Sodium dodecyl sulfate gels (5% acrylamide) of myofibrils at various stages of cross-linking digested with (a) chymotrypsin and (b) papain. Conditions for the cross-linking reaction are the same as in Figure 4.

cross-linked with DTBP and the resulting material examined in the phase-contrast microscope. No qualitative differences were observed between this system and the control (non-cross-linked) myofibrils except for the presence of aggregates formed as a result of interfibrillar cross-links. We also found that highly cross-linked myofibrils (DTBP, 0.3 mg/mL; cross-linking time, 16 h) are unable to undergo contraction in the presence of activating concentrations of MgATP and Ca ions.

Sodium dodecyl sulfate gels of the myofibrils cross-linked to various extents were examined to determine the pattern and degree of cross-linking. Results are presented in Figure 5. It is clearly seen that cross-linking of components of the thin filament complex is inefficient compared to that of myosin heavy chains.

Proteolytic Digestion of Cross-Linked Myofibrils. We monitored the cross-linking of various regions of the myosin molecule by digesting cross-linked myofibrils either with papain or chymotrypsin. Figure 6 shows sodium dodecyl sulfate gels of digested myofibrils at different extents of the cross-linking reaction. The sodium dodecyl sulfate gels of chymotrypsin-digested myofibrils (Figure 6a) and those of papain-digested myofibrils (Figure 6b) show very similar patterns except that chymotrypsin cleaves most of the myosin heavy chains without internal cleavage of the S-1 particle; on the other hand, proteolysis by papain results in a higher yield of fragmented particles. Neither papain nor chymotrypsin digested actin to a detectable extent. After the cross-linking reaction, the S-1 band as well as the rod band decreased in intensity, i.e., the head segments as well as the rod segments are cross-linked in the rigor myofibril.

Another important feature of the gels is that no new bands are seen as intermediate species. If we assume that myosin heads are cross-linked to a component of the thin filament complex (for example, to actin), a new band corresponding to the cross-linked complex of actin and S-1 (1:1 complex, since the rate of cross-linking of actin molecules is very slow as shown in Figure 5) should have appeared on the gels. This was not observed. On the contrary, cross-linking of myosin heads in the rigor myofibril appears to be coupled with the fast cross-linking reaction of the myosin rods as was found in the case of cross-linking synthetic thick filaments.

We also attempted to cross-link the rigor complex of S-1 (0.5 mg/mL) and the native thin filament (0.5 mg/mL) (see Materials and Methods) to provide additional evidence for the slow rate of cross-linking of myosin heads to the thin filament surface. Figure 7 shows that treatment of the complex with DTBP did not systematically reduce the intensities of the S-1 or actin bands, nor was a band attributable to covalently cross-linked acto-S-1 formed. The same result was also obtained when 1 mM $CaCl_2$ replaced EGTA, and when actin purified from an acetone powder was used in place of native thin filaments. Since a similar treatment of rigor myofibrils cross-linked almost 80% of the myosin heads, these findings support the conclusion that myosin heads are cross-linked to the thick filament surface much more rapidly than to the thin filament surface in the rigor myofibril.

The band pattern of the DTBP-treated complex of S-1 with

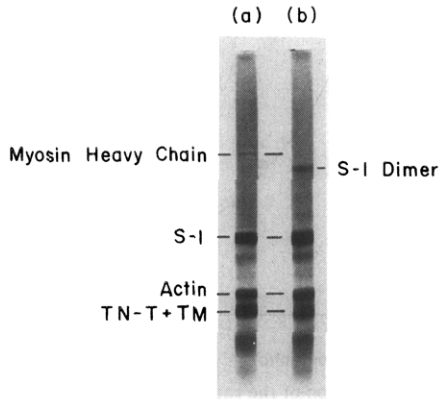

FIGURE 7: Sodium dodecyl sulfate gels (2.5% acrylamide–0.5% agarose) of the native thin filament–(S-1) complex (a) before and (b) after the cross-linking reaction. Solvent conditions for the cross-linking reaction: 80 mM KCl, 40 mM imidazole, and 1 mM EGTA (pH 7.4); cross-linker, DTBP (2.5 mg/mL); cross-linking time, 4 h at 4 °C. TN-T and TM refer to troponin-T and tropomyosin, respectively.

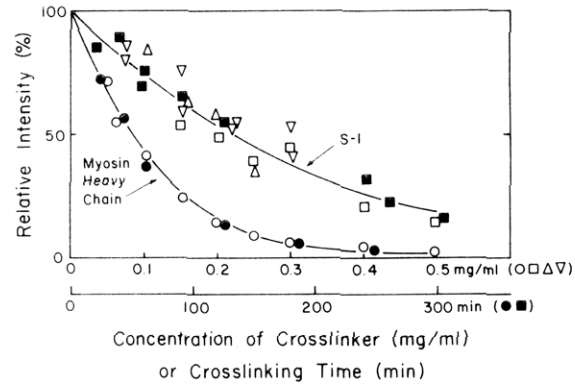

FIGURE 8: Kinetics of cross-linking myosin heavy chains and myosin heads in the rigor myofibril (open symbols) and in the synthetic thick filament (closed symbols). The relative intensities of the myosin heavy chain band (○,●), the S-1 band after papain digestion (□,■), and the S-1 band after chymotrypsin digestion ((△) 0.2 mg/mL for 10 min; (▽) 0.3 mg/mL for 15 min) are shown. Note that the abscissa scales are normalized so that the intensity change in the myosin heavy chain band follows a single decay curve in both cases. Conditions for cross-linking myofibrils: solvent, 80 mM KCl, 40 mM imidazole, and 1 mM EGTA (pH 7.4); cross-linker, DTBP; cross-linking time, 16 h at 4 °C. See Figure 2 for conditions of cross-linking synthetic thick filaments.

native thin filaments shows some minor differences from the untreated control (Figure 7). First, the myosin heavy chain band of the control (which is due to contamination in the native thin filament preparation) disappears, presumably to the top of the gel. Secondly, a faint band of apparent mol wt 180 000 is formed by DTBP treatment. Based on its molecular weight, we identify this species as a dimer of S-1, possibly formed by cross-linking of neighboring S-1 particles on the "decorated" thin filament complex.

Kinetics of Cross-Linking Myosin Heads to the Thick Filament Surface in the Rigor Myofibril. Myofibrils at various levels of cross-linking were digested either with papain or chymotrypsin and the change in the relative intensity of the S-1 band was followed by densitometry of sodium dodecyl sulfate gels (Figure 8). Digestion by papain gives the same DTBP concentration-dependent change in the relative intensity of the S-1 band (□) as digestion by chymotrypsin (△). The change in the relative intensity of the myosin heavy chain band on sodium dodecyl sulfate gels of myofibrils at various levels of cross-linking in the absence of proteolytic digestion was also followed as shown in the same figure (○).

To confirm our belief that the decrease in the relative intensity of the S-1 band during the cross-linking reaction stems from a gradual increase in cross-links between myosin heads and the thick filament surface, and not from a decrease in the ability of the enzyme to digest the myosin heavy chains, we digested myofibrils at higher concentrations of chymotrypsin (0.3 mg/mL) and for a longer period of time (15 min). The results (Figure 8 (▽)) show that the change in the conditions for proteolytic digestion had no effect on the change in the relative intensity of the S-1 band.

To compare the kinetics of cross-linking myosin heads in the synthetic thick filament and in the rigor myofibril, the time-dependent changes in the relative intensity of the heavy chain and S-1 bands shown in Figure 2 are replotted in Figure 8 (●, ■) by normalizing the abscissa scales (see Materials and Methods). This leads to the striking result (Figure 8) that the change in the relative intensity of the S-1 band during cross-linking synthetic thick filaments and rigor myofibrils fits a single decay curve.

Changes in Properties of Myosin during the Cross-Linking Reaction. The extent of the changes in functional properties of myosin induced by the cross-linking reaction was examined by measuring the ATPase activity and actin-binding capacity before and after the reaction. Cross-linking of synthetic thick filaments with IM-SH/H_2O_2 is a two-step process: the amino groups of the filaments are first reacted with IM-SH and then the cross-linking reaction to form a disulfide bridge is carried out in the presence of 10 mM H_2O_2. Under the conditions used for the modification of synthetic thick filaments, about 10% of the total amino groups react with this reagent (the number of free amino groups was determined by titration with trinitrobenzenesulfonic acid (Habeeb, 1966)), and the IM-SH-modified myosin retains 60% of the original EDTA–ATPase activity and shows an elevation to 160% of the original Ca-ATPase activity. After oxidation by H_2O_2 for 4 h, nearly 90% of the total heavy chains are cross-linked and the cross-linked filament exhibits 60% of the original EDTA–ATPase activity and 170% of the original Ca-ATPase activity. The cross-linking of filaments by DTBP is a one-step process. When 90% of the total heavy chains are cross-linked, 25% of the total amino groups are blocked, and the cross-linked filament shows 15% of the original EDTA–ATPase activity and 200% of the original Ca-ATPase activity.

To see if amidinated myosin can still bind to actin to form the rigor complex in the absence of MgATP, we examined the binding property of myosin heads in myofibrils after reaction with dimethyl malonimidate. Dimethyl malonimidate has only one methylene group between the two imidate groups. Although it reacts with lysine side chains, no cross-links are formed between peptide chains in myofibrils even after extensive amidination (Figure 9a).

Myofibrils before and after dimethyl malonimidate modification were digested by chymotrypsin and an aliquot of the digested material analyzed by sodium dodecyl sulfate gel electrophoresis (Figure 9b). Aliquots of digested material were centrifuged (5000 rpm for 10 min) in the presence and in the absence of MgATP (5 mM), and the supernatant was also analyzed by sodium dodecyl sulfate gel electrophoresis (Figure 9c and d). After extensive amidination by dimethyl malonimidate virtually all of the modified S-1 particles produced by chymotryptic digestion bind to actin and consequently spin down with myofibrils in the absence of MgATP, and almost all of them are released from actin and remain in the supernatant after the spin in the presence of MgATP.

Discussion

We observed a decrease in the yield of S-1 obtained by proteolytic digestion of synthetic thick filaments as a result of treatment by the cross-linking reagent, DTBP or IM-SH/H_2O_2, which we attribute to covalent bridging of myosin heads to the thick filament surface, i.e., to rod segments of neighboring myosin molecules in the thick filament backbone. This conclusion is supported by the following evidence.

First, cross-links between the two heads of the same myosin molecule were not detected even as an intermediate during the cross-linking reaction. Thus, the decrease in intensity of the S-1 band must result from the formation of cross-links between the individual heads and the rod segments of myosin. Secondly, the myosin head is apparently not cross-linked to its own rod segment for the following reasons. (1) The formation of cross-links showed no effect on the extent of proteolytic digestion of heavy chain (Figure 1). If cross-links had been formed at the sites of cleavage of these enzymes, some inhibition of enzymatic activity should have been observed through steric hindrance. (2) It is probable that the digestible segment in the junction between the rod and head segments of myosin involves a significant segment of polypeptide chain (this segment is digested both by papain and chymotrypsin which have differing specificities). On the other hand, the maximum length of the cross-link is 11–15 Å, and the actual length could be much shorter because of internal rotation around the C–C bond. Therefore, it is unlikely that the cross-links bridge the head and rod segments over the digestible area between them. (3) D'Albis and Gratzer (1976) have recently employed dithiobis(succinimidyl propionate) to cross-link myosin in high ionic strength solvent (0.5 M KCl) where myosin is in rapid, reversible monomer–dimer equilibrium (Godfrey and Harrington, 1970b). They found that myosin rod segments and head segments were readily cross-linked in this solvent to form rod dimers and head dimers while no cross-linking was observed between rod and head segments. The length of this cross-linker is the same as that of DTBP and it also bridges between lysine side chains. (4) Our recent (unpublished) cross-linking experiments on heavy meromyosin have also failed to reveal any significant formation of head–head or head–rodlet (S-2) cross-links. When heavy meromyosin was reacted with DTBP under conditions (see legend, Figure 2) which allow ~50% of the heads to be cross-linked to the thick filament surface, and the resulting product was digested with chymotrypsin, only trace amounts of bands corresponding to head–head or head–rodlet species were detected on sodium dodecyl sulfate gels.

X-ray diffraction studies have shown that the Brownian motion of cross-bridges (myosin heads) is restricted in living muscle. Any large motion of the cross-bridges would act to smear out the sharp axial reflections characteristic of the resting state. The fluorescence depolarization measurements of Mendelson et al. (1973) and Mendelson and Cheung (1976) have revealed that the rotational Brownian motion of myosin heads is also highly restricted in synthetic filaments and relaxed glycerinated myofibrils at neutral pH as compared to the mobility of the heads of individual myosin molecules. However, a large increase in rotational mobility of the heads of synthetic filaments was observed on raising the pH from 6.8 to 8.3 (μ = 0.12). Consistent with the depolarization studies, recent experiments in this laboratory reveal that the rate of cross-linking the head segments to the thick filament surface (normalized using the rate of cross-linking myosin heavy chains) is significantly reduced on elevating the pH of a synthetic filament solution from 7.4 to 8.3 (Chiao-Chen, 1977). Thus, the results

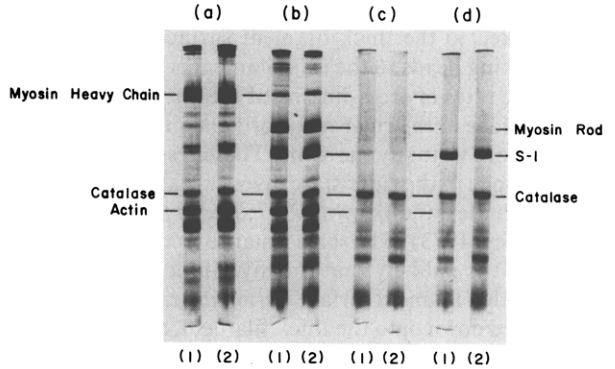

FIGURE 9: Sodium dodecyl sulfate gels (5% acrylamide) of (1) intact and (2) dimethyl malonimidate modified myofibrils (a) before and (b)–(d) after chymotryptic digestion; (b) total digest, (c) supernatant after centrifuging (5000 rpm, 10 min) in the absence of MgATP, and (d) supernatant after centrifuging (5000 rpm, 10 min) in the presence of 5 mM MgATP. Solvent conditions for the modification reaction: 80 mM KCl, 40 mM imidazole, and 1 mM EGTA (pH 7.4); dimethyl malonimidate, 2.5 mg/mL; reaction time, 4 h at 4 °C.

of the present study showing that the head segments are rapidly cross-linked to the thick filament surface at neutral pH seem to imply that the proximal surface of the head segment is in contact with the filament surface under these conditions.

The present study did not detect any change in the time course of cross-linking of myosin heads to the thick filament surface on the addition of Ca ions despite our expectation that the rate of cross-linking of myosin heads to the thick filament surface should be a sensitive indicator of changes in the radial disposition of the heads within the thick filament. It seems unlikely that chemical modification of myosin with the cross-linkers forced the heads to take a certain position regardless of the medium, since the (normalized) time courses of cross-linking with DTBP and IM-SH/H_2O_2 fit the same decay curve. Moreover, if we suppose that some type of denaturation of myosin is a prerequisite for cross-linking the heads, the time course should show a lag phase before an appreciable fraction of myosin heads is cross-linked. This behavior was not observed. It is also unlikely that the myosin head was drawn down onto the thick filament surface by first cross-linking the subfragment 2 (S-2) segment of the myosin rod to the thick filament surface since, as noted above, the rate of cross-linking the heads would be expected to exhibit a lag phase in the early stages of the reaction. Thus, the present study as well as the experiments of Nihei et al. (1974) and Mendelson and Cheung (1976) strongly suggest that Ca ions do not induce myosin heads to swing away from the thick filament surface. Nevertheless, it should be emphasized that the cross-linking method employed here cannot rule out an angular change in the orientation of myosin heads which would allow an appreciable area of the proximal surface of the head to remain in contact with the thick filament surface. The decrease in the myosin layer line intensity seen in x-ray diffraction studies of stimulated, highly stretched muscles (Haselgrove, 1970, 1975; Huxley, 1972) and the hydrodynamic measurements of native and synthetic thick filaments (Morimoto and Harrington, 1974b) may actually reflect an angular change in the orientation or a swivelling of myosin heads on binding Ca ions rather than a radial movement away from the thick filament surface. Such an azimuthal change in the orientation of the heads would not be expected to lead to an increase in rotational Brownian motion of the heads and would not therefore be incompatible with the fluorescence depolarization experiments of Mendelson and Cheung.

In the rigor myofibril almost all myosin heads are rapidly cross-linked to the thick filament surface while the rate of cross-linking heads to the thin filament is very slow. Furthermore, the rates of cross-linking of the heads to the thick filament surface in the rigor myofibril and in the synthetic thick filament are virtually the same. These results are quite unexpected, since in the rigor state myosin heads are currently believed (Huxley and Brown, 1967; Huxley, 1968; Haselgrove and Huxley, 1973) to be some distance from the thick filament surface. It is unlikely that the myosin heads are dissociated from the thin filament surface during the cross-linking reaction and drawn down onto the thick filament, since we found that even after extensive amidination by dimethyl malonimidate the heads are still able to bind to actin to form the rigor complex in the absence of MgATP. It is also unlikely that myosin heads are zippered down through formation of cross-links between the S-2 segment and the thick filament surface in view of the argument presented earlier. Thus, it seems reasonable to conclude that in all stages of the reaction, myosin heads in the rigor myofibril remain locked to both thin and thick filaments and are cross-linked to the thick filament surface much faster than to the thin filament surface. The simplest explanation for this finding is that the contact surface area between the proximal surface of the myosin head and the thick filament surface is appreciably greater than that between the myosin head and the thin filament surface. Another possibility is that the lysine side-chain distribution at the head–actin interface is inappropriate for cross-linking.

As we have noted earlier, the myosin projections from the three- or four-stranded thick filament models of Squire in a relaxed muscle probably extend to a radius of 180–200 Å from the core of the thick filament (Squire, 1972, 1975) while the surface of the actin filaments is at a radius of 180–190 Å (Huxley and Brown, 1967). Recent measurements of subfragment 1 in solution provide a radius of gyration for this particle of ~32 Å which is consistent with a prolate ellipsoid of long axis 120–150 Å (Kretzschmar et al., 1976); the image reconstruction studies of Moore et al. (1970) indicate a maximum thickness of 50 Å and length of about 120 Å for the cross-bridge. If we assume that the bridges are arranged systematically along the surface of the filament core according to a three- or four-stranded Squire model, the lengths of the S-1 moieties would seem to be sufficient to span the interfilament distance in the resting state of muscle.

We have assumed in the above discussion that the filament lattice of glycerinated myofibrils remains invariant during the cross-linking reaction. Any significant decrease in interfilament spacing could lead to contact between the proximal surface of the myosin head and the thick filament surface. In this case, however, we would expect a lag phase in the early stages of the head–rod linkage reaction. Although such a lag phase was not observed in our experiments (see Figure 8), it will be worthwhile in later studies to look for changes in the interfilament spacing by following the equatorial x-ray reflections during cross-linking of glycerinated fibers in rigor to confirm this point.

With this cautionary note, we conclude from the results of the present study that neither the presence of Ca ions nor the shift from resting to rigor state (at rest length) results in release of myosin heads from the thick filament surface. This conclusion provides support for the recent proposal of Lymn and Cohen (1976) that the change in the ratio ($I_{1,1}/I_{1,0}$) of the two principal equatorial x-ray reflections observed when muscle contracts at a constant length or goes into rigor may stem from an azimuthal rather than an outward radial movement of cross-bridges.

Note Added in Proof

Using x-ray diffraction, Drs. John Haselgrove and John Murray (MRC Laboratory of Molecular Biology, Cambridge) have now kindly measured the lattice spacing of cross-linked and non-cross-linked glycerinated myofibrils. Bundles of rabbit psoas muscle about 1 mm in diameter were cross-linked with DTBP (0.5 mg/mL) in 80 mM KCl, 40 mM imidazole, and 1 mM EGTA (pH 7.4) overnight at 4 °C. Based on sodium dodecyl sulfate gel electrophoresis, 80% of the myosin heavy chains were cross-linked under these conditions. As a control, muscle bundles from the same preparation were soaked overnight in the same buffer in the absence of DTBP. The equatorial reflections showed no difference in lattice spacing between the cross-linked and non-crossed-linked samples. Therefore, we can rule out the possibility that cross-linking of myosin heads to the thick filament surface in the rigor myofibril results from a contraction in the lattice spacing during the cross-linking reaction.

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Calcium Binding Site of Trypsin as Probed by Lanthanides†

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ABSTRACT: A number of physicochemical techniques have been applied to identify, locate, and characterize the Ca^{2+}-binding site in porcine and bovine trypsin. The fluorescent lanthanide Tb^{3+} and the paramagnetic lanthanide Gd^{3+} were used as probes for the Ca^{2+}-binding site in the trypsin molecule. The fluorescent lanthanide Tb^{3+} was found to bind to the specific Ca^{2+}-binding site on the trypsin molecule concomitant with a large increase in its fluorescence. The pH dependence of the Tb^{3+}-binding process to the trypsin molecule and studies on proton release upon Tb^{3+} binding to the protein reveal the involvement of two carboxyl residues in metal binding. Proton relaxation rate measurements on the Gd^{3+}-trypsin complex reveal that upon metal binding six of the eight water molecules coordinating the aquo-Gd^{3+} ion are released where two water molecules remain bound to the protein-bound Gd^{3+} ion. Model building of the trypsin molecule identifies the two carboxylate residues at the metal-binding site as Glu-70 and Glu-80, as recently revealed by x-ray crystallographic studies. Fluorescence excitation studies on the trypsin-Tb^{3+} complex reveal energy transfer from a tryptophan residue to the bound Tb^{3+} ion. This residue is identified as Trp-141. Trypsin was also found to possess a low-affinity site for lanthanide ions which is incapable of binding Ca^{2+}. The existence of a secondary lanthanide-binding site is responsible for the variation of the circular polarized luminescence spectrum of the Tb^{3+}-trypsin complex with the Tb^{3+} to protein ratio. Some differences are found between the spectroscopic properties of the lanthanide complexes of bovine trypsin and porcine trypsin. These differences stem from the structural differences of the Ca^{2+}-binding sites of the two types of trypsins.

Calcium binding to trypsin is known to inhibit the enzymatic autodigestion of the protein (Buck et al., 1962). This effect seems to underlie the biological function of calcium in this system and there has been considerable interest in locating the calcium site on the enzyme (Stroud et al., 1971, 1974; Abbott et al., 1975a,b; Bode and Schwager, 1975a,b). In a previous communication, we have shown that the trivalent lanthanide ions as well as Mn^{2+} and Cd^{2+} are capable of binding at the single Ca^{2+}-binding site of porcine trypsin (Epstein et al., 1974). Using the fluorescence enhancement of Tb^{3+}, the electron spin resonance spectrum of Mn^{2+}, and the radioactive $^{169}Yb^{3+}$ isotope, the stoichiometry and association constants of the cation-trypsin complexes were determined. These studies are now extended to include measurements of water proton relaxation rates, proton release, and circularly polarized luminescence with porcine trypsin as well as some measurements with bovine trypsin. The present work involves the determination of the number of carboxylate ligands at the binding site, the determination of the number of water molecules coordinated to the bound Gd^{3+} ion, and the identification of the chromophore involved in the energy transfer that leads to fluorescence enhancement of bound Tb^{3+}. Our results, viewed in conjunction with the molecular model of trypsin,

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