Evidence for the Association between Two Myosin Heads in Rigor Acto–Smooth Muscle Heavy Meromyosin†

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ABSTRACT: The rigor complexes that formed between rabbit skeletal muscle F-actin and chicken gizzard heavy meromyosin (HMM), in which the heavy chains had been cleaved with trypsin into 24K, 50K, and 68K fragments, were examined by using the zero-length chemical cross-linker 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDC). Two cross-linked products of approximate M_r 115K and 60K were generated. These products were not obtained by EDC treatment of HMM in the absence of F-actin. The HMM fragments that participated in cross-linking were identified by fluorescent labeling and amino acid composition studies. The 115K peptide was determined to be a covalently cross-linked complex that formed between actin and the COOH-terminal 68K fragment of the HMM heavy chain. Our results are in agreement with a previous study which proposed that the site of cross-linking between HMM and F-actin resides within the COOH-terminal 22K fragment of the myosin subfragment 1 heavy chain [Marianne-Pêpin, T., Mornet, D., Bertrand, R., Labbé, J.-P., & Kassab, R. (1985) *Biochemistry 24*, 3024–3029]. The 60K peptide, however, was not a product of cross-linking between HMM and F-actin. On the basis of its amino acid composition, we concluded that this 60K peptide was a cross-linked dimer of the NH_2-terminal 24K fragments of the HMM heavy chain. The cross-linking of acto–gizzard HMM significantly increased the Mg-ATPase activity of gizzard HMM without any observable phosphorylation of the regulatory (20K) light chains. These findings suggest that, in the rigor complex, the two heads of gizzard HMM are in contact with each other and that the Mg-ATPase activity of HMM is enhanced by an irreversible occupation of the actin-binding site on HMM.

With the exception of *Acanthamoeba* myosins IA and IB (Pollard & Korn, 1973), all myosins so far reported have two globular heads and a rod-shaped tail. The globular heads can be separated from the tail by using proteolytic enzymes. Each head fragment retains the functional properties of the native myosin molecule (Lowey et al., 1969). For example, fluorescent actin filaments can translocate on a glass slide coated with single-headed myosin molecules (Yanagida et al., 1984; Yano-Toyoshima et al., 1987). Since this study and other studies have shown that single myosin heads are both enzymatically active and capable of generating motile force, it is not yet clear why a native myosin molecule has two heads.

A more complete understanding of the interface region between myosin heads and F-actin is essential for elucidating the molecular mechanism of the sliding of myosin heads along actin filaments. Recent studies have examined this region using the techniques of limited proteolysis and chemical cross-linking with bifunctional reagents. It was found that limited digestion of rabbit skeletal muscle myosin subfragment 1 (S-1)[1] with trypsin generated three major heavy-chain fragments of approximately 25K, 50K, and 20K (Bálint et al., 1978). Experiments with zero-length cross-linkers have also indicated that the 50K and the 20K fragments cross-link actin at independent sites on the S-1 molecule (Mornet et al., 1981; Sutoh, 1982, 1983). Since the two binding sites were found to be in close proximity to each other in the myosin heavy chain sequence, Sutoh suggested that they are also spatially close to each other in the S-1 molecule and that they form an actin-binding region. Similar results were obtained in a study using smooth muscle myosin S-1, although proximity of the smooth muscle 50K region to F-actin was not demonstrated (Marianne-Pêpin et al., 1985). Although the actin-binding site has been well characterized, thus far there have been no studies of the relationship between the two myosin heads that attach to F-actin.

In this study, the zero-length cross-linker 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDC) was used to examine whether or not a head-to-head interaction occurs when gizzard HMM binds to F-actin. In an attempt to answer this question, we treated the actin–gizzard HMM complex with EDC. In order to identify the postulated head-to-head interaction sites within the HMM molecule, the heavy chains of gizzard HMM were cleaved with trypsin into 24K, 50K, and 68K fragments prior to the cross-linking reaction. These three heavy chain fragments remain associated under nondenaturing conditions, and HMM cleaved in this way retains functional activities. Our results demonstrate not only that gizzard HMM cross-links with F-actin through the COOH-terminal 68K fragment region but also that the two heads of an HMM molecule cross-link to each other via their

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[1] Abbreviations: HMM, heavy meromyosin; S-1, subfragment 1; ATPase, adenosine 5′-triphophatase; IAEDANS, *N*-(iodoacetyl)-*N*′-(5-sulfo-1-naphthyl)ethylenediamine; AEDANS, *N*-acetyl-*N*′-(5-sulfo-1-naphthyl)ethylenediamine; DACM, *N*-[7-(dimethylamino)-4-methyl-3-coumarinyl]maleimide; EDC, 1-ethyl-3-[3-(dimethylamino)propyl]-carbodiimide; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; DTT, dithiothreitol; $NaDodSO_4$, sodium dodecyl sulfate.

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NH_2-terminal 24K fragment regions.

Materials and Methods

Preparation of Proteins. Chicken gizzard myosin was prepared as reported by Ikebe et al. (1978). Rabbit skeletal muscle actin was isolated by the procedure of Spudich and Watt (1971).

IAEDANS Labeling of Myosin. Gizzard myosin was dissolved in a solution containing (a) 1.5 mM ATP, 0.15 M KCl, 1 mM $MgCl_2$, and 20 mM Tris-HCl (pH 7.5); (b) 0.15 M KCl, 10 mM $MgCl_2$, and 20 mM Tris-HCl (pH 7.5); or (c) 0.15 M KCl, 10 mM EDTA, and 20 mM Tris-HCl (pH 7.5). Different amino acids within the primary sequence of gizzard myosin were labeled with *N*-(iodoacetyl)-*N'*-(5-sulfo-1-naphthyl)ethylenediamine (IAEDANS) by using several different labeling conditions. Labeling was performed at 20 °C by incubating myosin (5 mg/mL) with an 80 molar equiv of IAEDANS. At appropriate times (30 s–60 min) the reaction was stopped by the addition of 30 mM 2-mercaptoethanol.

Tryptic Cleavage of AEDANS-Labeled Myosin and Isolation of HMM. Following IAEDANS labeling, gizzard myosin (2.5 mg/mL) in 0.3 M KCl, 2 mM $MgCl_2$, 0.3 mM DTT, and 20 mM Tris-HCl (pH 7.5) was digested with trypsin (weight ratio: protease/myosin = 1/250) for 15 min at 20 °C. Proteolytic digestion was terminated by adding trypsin inhibitor (weight ratio: inhibitor/protease = 2/1). HMM was then isolated according to the method of Maita et al. (1987). The HMM heavy chains thus obtained consisted of three major fragments of approximate molecular weights 24K, 50K, and 68K as previously described (Onishi & Watanabe, 1985).

DACM Labeling of F-Actin. Fluorescent F-actin was prepared as described by Yamamoto et al. (1977). Briefly, rabbit skeletal muscle F-actin (2 mg/mL) was dialyzed overnight against 0.3 mM ATP, 50 mM KCl, 2 mM $MgCl_2$, 0.2 mM $CaCl_2$, and 2 mM Tris-HCl (pH 8.0) and then reacted with a 1.7 molar equiv of *N*-[7-(dimethylamino)-4-methyl-3-coumarinyl]maleimide (DACM) for 45 min at 0 °C. The reaction was quenched by adding 30 mM 2-mercaptoethanol.

Cross-Linking of the Actin–HMM Complex or HMM. Gizzard HMM (0.5 mg/mL) was mixed with skeletal muscle F-actin (weight ratio: actin/HMM = 1) in 0.1 M KCl, 10 mM $MgCl_2$, and 10 mM imidazole hydrochloride (pH 7.0). The mixture was dialyzed overnight against the same solution to remove the free ATP present in the original stock solution of F-actin. In the acto–HMM complex, either the actin was labeled with DACM or the HMM was labeled with IAEDANS. The cross-linking reaction was initiated by adding a 5% volume of freshly prepared 0.1 M EDC in 0.1 M imidazole hydrochloride (pH 7.0) to the acto–HMM solution. The reaction was allowed to proceed for 1 or 2 h at 24 °C and then terminated by the addition of 30 mM 2-mercaptoethanol. AEDANS-modified gizzard HMM in the absence of F-actin was also treated with EDC as described for the actin–HMM complex.

Gel Electrophoresis. Aliquots from the cross-linking reaction mixtures were submitted to electrophoresis in the presence of $NaDodSO_4$ (Laemmli, 1970), using polyacrylamide slab gels containing 11% acrylamide and 0.3% bis(acrylamide). Fluorescent bands were observed under a long-wavelength ultraviolet lamp before staining the gel with Coomassie brilliant blue.

ATPase Activity. After an hour of reaction, an aliquot (0.1 mL) from each cross-linking reaction mixture was diluted 10 times with the assay medium, and the Mg-ATPase activity was determined at 37 °C. The assay medium consisted of 50 mM KCl, 10 mM $MgCl_2$, 0.2 mM $CaCl_2$, 0.3 mM DTT, 20 mM Tris-HCl (pH 7.5), and 1 mM ATP. The reaction was stopped by adding trichloroacetic acid to make a final concentration of 7%. Inorganic phosphate (P_i) was measured colorimetrically according to the method of Youngburg and Youngburg (1930).

S-Carboxymethylation of the Cross-Linked Product. Under a nitrogen barrier, the cross-linked acto–HMM complex (600 mg at a weight ratio of actin/HMM = 1/4) in 40 mL of a denaturing solution containing 6 M guanidine hydrochloride, 5 mM EDTA, and 0.5 M Tris-HCl (pH 8.0) was reduced with a 100-fold molar excess of 2-mercaptoethanol for 4 h at 37 °C. The amount of 2-mercaptoethanol was 100-fold greater than the calculated number of HMM cysteine residues. A freshly prepared solution of iodoacetic acid at an equimolar concentration to the 2-mercaptoethanol was added to the reaction mixture as described by Crestfield et al. (1963). The reaction was allowed to proceed for 1 h at 37 °C. The S-carboxymethylated acto–HMM complex was then submitted to gel filtration on a Sephadex G-100 column equilibrated with a solution containing 5 M guanidine hydrochloride, 20 mM Tris-HCl (pH 8.0), and 1 mM EDTA.

Amino Acid Composition. Peptide samples were hydrolyzed at 110 °C under vacuum in 5.7 N HCl with 1% phenol for 20 h and then derivatized with phenyl thiocyanate in a Waters Pico-Tag work station (Millipore Co.). Amino acid derivatives were analyzed by reverse-phase high-performance liquid chromatography using a Waters Pico-Tag column (3.9 × 150 mm).

Protein Concentrations. Protein concentrations were determined by measuring protein absorbance according to the biuret method (Gornall et al., 1949) and then standardizing these values according to the micro-Kjeldahl method (Ballentine & Gregg, 1947).

Results

Cross-Linking of Acto–HMM Complexes. EDC was added to rigor complexes formed between F-actin and AEDANS-modified derivatives of tryptic gizzard HMM in order to induce cross-linking, and the products were analyzed by gel electrophoresis in the presence of $NaDodSO_4$. The protein band patterns in (A) of Figure 1 show that two new polypeptide species were produced during treatment of the rigor complex with EDC for 1 or 2 h. The molecular weights of these two species were estimated by using standard protein markers and were found to be approximately 115K and 60K. As previously described and shown in Figure 1 [lane 0 of (A)], the tryptic HMM derivative consisted of five components: three heavy chain fragments of 24K, 50K, and 68K, the 17K light chain (L_{17}), and a fragment of the 20K light chain (L'_{20}). The fluorescence profiles illustrated in (B) of Figure 1 indicate that fluorescent labels were incorporated into the newly formed 60K species. This result suggests that the 60K species contains the 24K fragment and/or the 17K light chain as a component, since the AEDANS-modified HMM derivative used in this experiment also had strongly fluorescent 17K and 24K bands.

The results of the EDC treatment of AEDANS-modified gizzard HMM in the absence of F-actin are shown in (C) and (D) of Figure 1. A faint protein band appeared around 115K after treatment with EDC for 1 or 2 h. Since this band migrated slightly faster than the 115K species produced by the EDC treatment of the acto–HMM complex, it appears to be a different species. Neither the Coomassie-stained (C) nor the fluorescence profile (D) revealed the presence of a 60K band. These results suggest that the formation of rigor com-

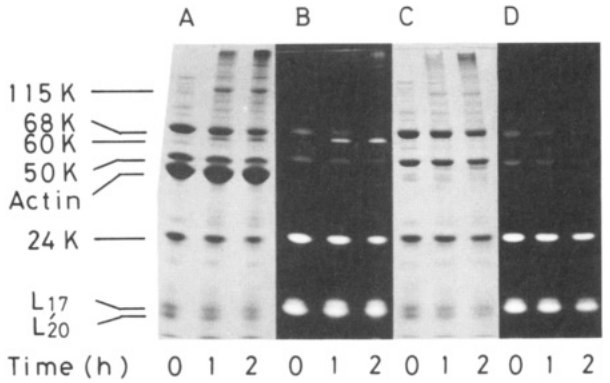

FIGURE 1: Carbodiimide-catalyzed covalent cross-linking of the rigor complex formed between F-actin and AEDANS-modified gizzard HMM. The IAEDANS labeling was carried out by incubating gizzard myosin (5 mg/mL) with an 80 molar equiv of IAEDANS in 1.5 mM ATP, 0.15 M KCl, 1 mM $MgCl_2$, and 20 mM Tris-HCl (pH 7.5) for 50 min at 20 °C. The rigor complex (1 mg/mL) between skeletal muscle F-actin and AEDANS-modified gizzard HMM (at a weight ratio of actin/HMM = 1) was reacted with EDC as specified under Materials and Methods. At time 0 h (not cross-linked) and at 1 and 2 h following cross-linking, a protein aliquot was withdrawn and mixed with excess 2-mercaptoethanol to terminate the cross-linking reaction. Protein samples (10 μg of HMM) were submitted to gel electrophoresis in the presence of $NaDodSO_4$. The Coomassie blue staining pattern (A) and the fluorescence profile (B) of the same gel are shown. 24K, 50K, and 68K indicate the tryptic fragments of the HMM heavy chain. L_{17} and L'_{20} indicate the 17K light chain of HMM and the tryptic fragment of the HMM 20K light chain, respectively. 60K and 115K indicate two cross-linked species. The 60K band was fluorescent, whereas the 115K band was not fluorescent. EDC treatment of AEDANS-modified gizzard HMM in the absence of F-actin was performed as the control. Polypeptide bands stained with Coomassie blue (C) and the fluorescent profile of the same gel (D) are illustrated.

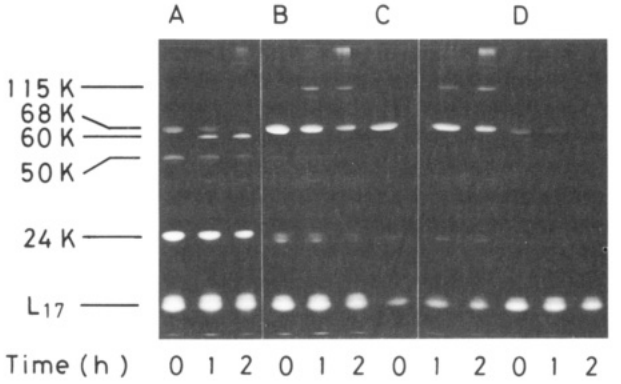

FIGURE 2: Identification of the gizzard HMM components that participate in carbodiimide-catalyzed cross-linking. Four AEDANS-modified derivatives of gizzard myosin were obtained by reacting gizzard myosin (5 mg/mL) with an 80 molar equiv of IAEDANS in 1.5 mM ATP, 0.15 M KCl, and 1 mM $MgCl_2$ for 90 min (A), in 0.15 M KCl and 10 mM $MgCl_2$ for 30 min (B), in 0.15 M KCl and 10 mM $MgCl_2$ for 30 s (C), and in 0.15 M KCl and 10 mM EDTA for 8 min (D). HMM was isolated from each fluorescent derivative of gizzard myosin and mixed with skeletal muscle F-actin. Conditions for the cross-linking reaction with EDC and for $NaDodSO_4$ gel electrophoresis were the same as described in Figure 1. The fluorescence profiles of the gels are shown. The NH_2-terminal 24K fragment of the HMM heavy chain became labeled with fluorescent AEDANS only in the presence of Mg-ATP (A). The 60K cross-linked product, the 24K homodimer, was fluorescent only in (A). On the other hand, fluorescent labeling of the COOH-terminal 68K fragment of the HMM heavy chain (B and C) was Mg-dependent; however, labeling did not occur in the presence of Mg-ATP (A) or in the presence of EDTA (D). The 115K band, the cross-linked product of the 68K fragment and actin, showed the same fluorescence pattern as the 68K fragment.

plex between F-actin and HMM is required for the production of the two cross-linked species of 115K and 60K.

To identify the fragmented HMM components that are involved in cross-linking HMM with F-actin or within the HMM molecule itself, cross-linking experiments were performed with four different AEDANS-modified derivatives of tryptic gizzard HMM. Their fluorescence profiles were visualized by $NaDodSO_4$ gel electrophoresis and were found to differ from each other (Figure 2). The Coomassie blue stained protein band patterns of all the derivatives were similar to the pattern shown in Figure 1A. The distribution of fluorescence in the cross-linked species depended upon which AEDANS-modified derivative was used in an experiment. For example, both the 60K and 24K bands were fluorescent in derivative A, but not in derivatives B–D. Since the 60K band was fluorescent only when the 24K band was fluorescently labeled, it appears that the 24K fragment is a component of the 60K species. In contrast, the 115K band was fluorescent in (B) and (C), but this band was not fluorescent in (A) and (D). The fluorescence intensity of the 17K band was much weaker in (C) than in (B), although the 115K band showed a similar fluorescence intensity in both (B) and (C). This result suggests that the 17K light chain is not a precursor of the 115K species. As the 68K bands showed approximately equal fluorescence intensities in (B) and (C), we conclude that the 68K fragment is a component of the 115K species.

Fluorescent F-actin was used to investigate whether or not the 115K and 60K species contained actin. Protein band patterns in (A) of Figure 3 indicated that both the 115K and 60K protein bands appeared during treatment with EDC. The 115K band was fluorescent, although the 60K band was not (Figure 3B), suggesting that the 115K species, but not the 60K species, contains actin. The same two faintly fluorescent

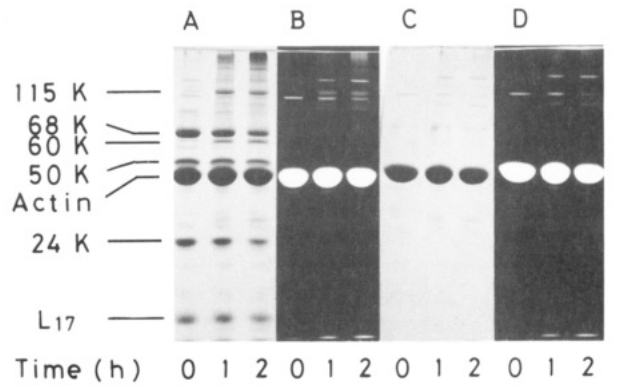

FIGURE 3: Identification of an actin-containing band. The rigor complex (1 mg/mL) between DACM-modified fluorescent F-actin and gizzard HMM at a weight ratio of actin/HMM = 1 was reacted with EDC as specified under Materials and Methods. At time 0 h (not cross-linked) and at 1 and 2 h following cross-linking, a protein aliquot was withdrawn and mixed with excess 2-mercaptoethanol to terminate the cross-linking reaction. Conditions for $NaDodSO_4$ gel electrophoresis were the same as in Figure 1. The protein banding pattern (A) and the fluorescence profile (B) of the same gel are shown. The 115K protein band was fluorescent, whereas the 60K protein band was not fluorescent. As a control, fluorescent F-actin was treated with EDC. The 115K band is not seen by Coomassie blue staining (C) nor is it by fluorescence (D). The higher M_r bands of approximately 100K and 150K (B and D) are not products of the F-actin–HMM cross-linking reaction, as they appeared when F-actin alone was treated with EDC (D).

components observed in (B) were also observed when F-actin alone was treated with EDC (Figure 3C,D). It is possible that trace amounts of α-actinin were a contaminant in our F-actin preparation. Since the two faintly fluorescent components migrated to approximately 100K and 150K, it is likely that they are α-actinin and the cross-linked complex of actin and α-actinin, respectively. Since these two bands appeared after

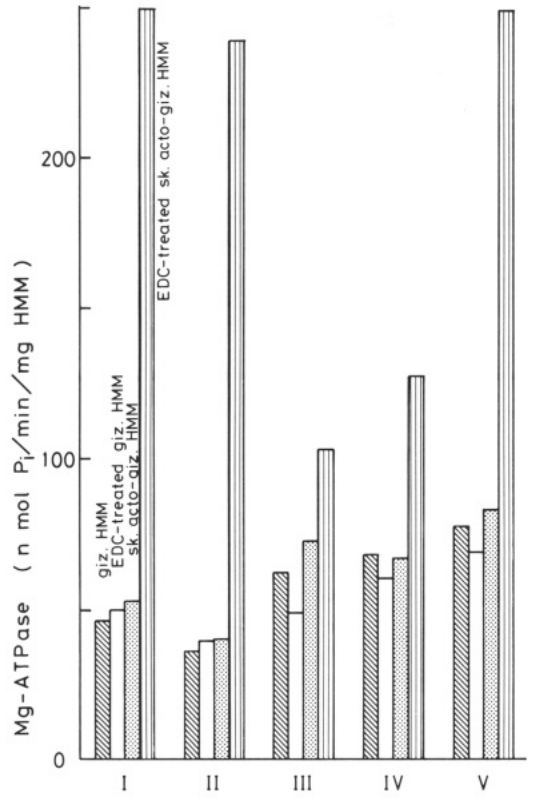

FIGURE 4: Effects of carbodiimide-catalyzed cross-linking on the Mg-ATPase activities of either acto–tryptic gizzard HMM or gizzard HMM. Conditions for the IAEDANS labeling of myosin in (I) were the same as described in Figure 1, and labeling conditions for (II), (III), (IV), and (V) were the same described for (A), (B), (C), and (D) in Figure 2, respectively. The EDC-initiated cross-linking reaction was performed as described in Figure 1 and was terminated at 1 h. Samples containing 0.05 mg of HMM were submitted to Mg-ATPase assays. Abbreviations: giz. HMM, gizzard HMM; EDC-treated giz. HMM, gizzard HMM that was cross-linked with EDC; sk. acto–giz. HMM, a mixture of skeletal muscle F-actin and gizzard HMM; EDC-treated sk. acto–giz. HMM, the cross-linked product of the rigor complex between skeletal muscle F-actin and gizzard HMM.

EDC treatment of F-actin alone, we can rule out the possibility that they are products of rigor complex formation.

Increased Mg-ATPase Activity of Cross-Linked Acto–HMM. Gizzard S-1 Mg-ATPase, with or without phosphorylatable (20K) light chain, is fully activatable by actin, whereas light chain depleted tryptic gizzard HMM is not activatable by actin (Nath et al., 1982). Since the EDC-catalyzed cross-linking of gizzard S-1 to F-actin stimulates Mg-ATPase activity of the acto–S-1 complex (Marianne-Pépin et al., 1985), we investigated whether or not the Mg-ATPase activity of light chain depleted tryptic HMM was affected during cross-linking to F-actin. Figure 4 shows that the Mg-ATPase activity of each acto–tryptic gizzard HMM sample is enhanced following EDC treatment, although the level of enhancement depended upon which AEDANS-modified HMM derivative was used (I–V; see figure legend for detail). These results suggest that the irreversible cross-linking of HMM with F-actin stimulates the Mg-ATPase activity of HMM. The degree of Mg-ATPase enhancement also depended upon the state of the 68K fragment. As illustrated in Figure 4, parts III and IV, a significant drop in Mg-ATPase activity occurred in the skeletal acto–gizzard HMM derivatives whose 68K fragment had been modified with IAEDANS.

Isolation of the 60K Cross-Linked Product. As we have described (see Cross-Linking of Acto–HMM Complexes), one precursor of the 60K cross-linked product appears to be the NH_2-terminal 24K fragment of the HMM heavy chain. We would expect there to be a second component, which is the counterpart of the 24K fragment. However, we were unable to identify a second component by gel analysis. We thus planned to isolate the 60K peptide from the cross-linking reaction mixture and then to identify the unknown precursor by its amino acid composition. Gizzard HMM, of which the 24K fragment was labeled with IAEDANS, was prepared as described in the legend for Figure 1. The rigor complex (2.5 mg/mL) which formed between the fluorescent HMM derivative and F-actin (weight ratio: actin/HMM = 1/4) in 0.1 M KCl, 10 mM $MgCl_2$, and 10 mM imidazole hydrochloride (pH 7.0) was reacted with 5 mM EDC for 2 h at 24 °C. The reaction was stopped by the addition of excess 2-mercaptoethanol. Saturated ammonium sulfate was added to the reaction mixture, and the fraction that precipitated at 67% saturation was collected. Denaturation and the S-carboxymethylation of the rigor complex were carried out as described (see Materials and Methods). The S-carboxymethylated actin–HMM sample (Figure 6, lane A) was subjected to gel filtration on a Sephadex G-100 column. The elution profile is shown in Figure 5A. We analyzed the polypeptides present in the various fractions by $NaDodSO_4$ gel electrophoresis and found that the 115K and 60K cross-linked products, the 68K and 50K fragments, and actin were contained in the first peak (Figure 6, lane B), whereas the 24K fragment with traces of the 50K fragment and actin were contained in the second peak (Figure 6, lane C).

The 60K-rich fractions including fractions 57–64 (peak I in Figure 5A) were pooled and rechromatographed on a CM-52 cellulose column (Figure 5B). Analysis of fractions by $NaDodSO_4$ gel electrophoresis indicated that the 60K species was eluted with an approximate NaCl concentration of 0.1 M (Figure 6, lane D). The 60K species could be separated from actin (the first peak), the 68K fragment (the shoulder of the second peak), and the 50K fragment (which was retained in the column) by this chromatography. The 60K species pooled from fractions 43–49 was further purified by fast protein liquid chromatography on a Mono Q column (Figure 5C). All of the fractions eluted with NaCl concentrations between 0.16 and 0.21 M contained the 60K species as the major component, although the earlier fractions of the peak were contaminated with trace amounts of an unidentified 88K component. Therefore, we pooled later fractions (elution volume 19.0–20.5 mL) and used them as the purified 60K peptide (Figure 6, lane E).

The 24K-rich fractions which included fractions 70–78 in Figure 5A (peak II; Figure 6, lane C) were subjected to chromatography on a CM-52 cellulose column (Figure 5D). Purified 24K polypeptide was obtained from fractions 48–52 (Figure 6, lane F).

The amino acid compositions of the 24K and 60K polypeptides were determined, and we found that they were extremely similar to each other (Table I). Since the amino acid sequence of chicken gizzard myosin is known (Onishi et al., 1986; Maita et al., 1987; Yanagisawa et al., 1987), the amino acid composition for each of the tryptically digested components of gizzard HMM could also be determined. The composition of each HMM component, with the exception of the 24K component, differed markedly from the observed composition of the 60K peptide. We thus concluded that the 60K species is a cross-linked homodimer of the NH_2-terminal 24K fragments of the HMM heavy chain.

Discussion

Certain aspects of inter- and intramolecular interaction between skeletal F-actin and gizzard HMM in the rigor

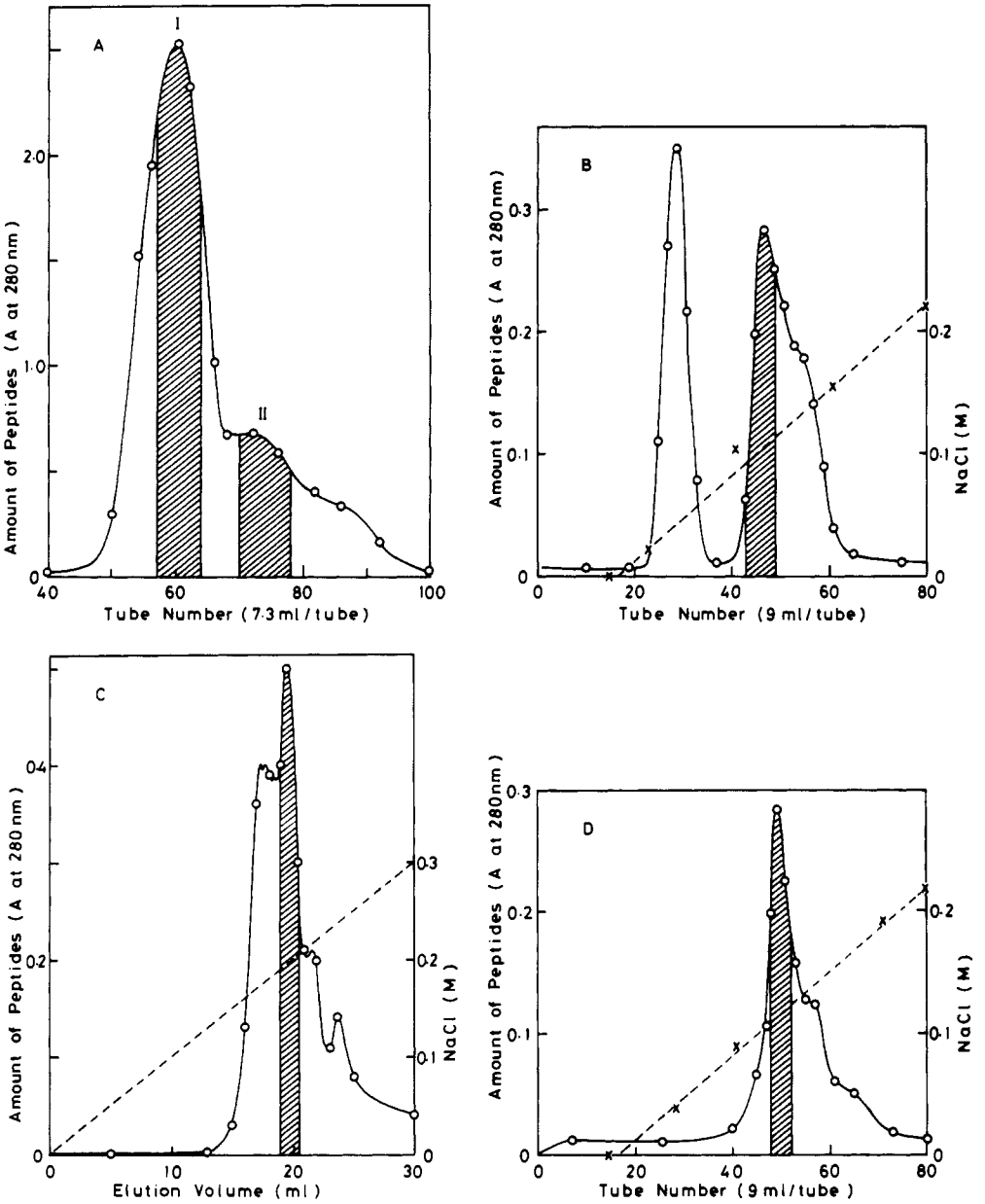

FIGURE 5: Separation of the 60K and 24K peptides from the carbodiimide-catalyzed cross-linked products. (A) Gel filtration. The S-carboxymethylated derivative of cross-linked acto–HMM (about 600 mg) was applied to a Sephadex G-100 column (5 × 95 cm) that had been equilibrated with 5 M guanidine hydrochloride, 20 mM Tris-HCl (pH 8.0), and 1 mM EDTA. Peptides were eluted with the same buffer at a flow rate of 1.2 mL/min. (B) Separation of the 60K cross-linked product from actin and from the 68K and 50K fragments of the HMM heavy chain by CM-52 column chromatography. The sample within the hatched area of the first peak in (A) was applied to a CM-52 cellulose column (2.3 × 35 cm) that had been equilibrated with a denaturing buffer containing 8 M urea and 50 mM sodium acetate (pH 5.0). Peptides were eluted with a linear gradient of NaCl concentrations from 0 to 0.5 M. The flow rate was 0.9 mL/min. (C) Purification of the 60K peptide by Mono S column chromatography. One-third of the total sample represented by the hatched area in (B) was applied to a Mono S HR 5/5 column (0.5 × 5 cm) that had been equilibrated with denaturing buffer as described in (B). Peptides were eluted with a linear gradient of NaCl concentrations from 0 to 0.3 M for 30 min at a flow rate of 1 mL/min. (D) Purification of the 24K peptide by CM-52 column chromatography. The sample represented by the hatched area in peak II of (A) was applied to the column. Conditions for the chromatography are described in (B).

complex were investigated by using the zero-length cross-linker EDC. Our results demonstrate that the two heads of an HMM molecule which attach to F-actin are also in contact with each other (Figure 7). This is the first demonstration of an interaction between the two HMM heads bound to F-actin in the rigor complex.

Two cross-linked products of M_r 115K and 60K were obtained by the reaction of EDC with the acto–HMM complex (see Figure 1). Experiments using either the combination of actin and fluorescent HMM (see Figure 2) or fluorescent actin and HMM (see Figure 3) revealed that the 115K product is the cross-linked product of actin and the COOH-terminal 68K fragment of the HMM heavy chain. Therefore, gizzard HMM heads appear to associate with F-actin through their 68K segment regions (arrows in Figure 7). Addition of papain to tryptically digested gizzard HMM resulted in cleavage of the 68K fragment into two components of 22K and 36K. Our results are in agreement with a previous study by Marianne-Pépin et al. (1985) in which the COOH-terminal 22K segment region of the S-1 heavy chain was shown to be involved in the EDC-catalyzed cross-linking of gizzard S-1 to F-actin. Although direct contact of the 50K segment region of skeletal muscle S-1 with F-actin was previously demonstrated by cross-linking experiments with EDC (Mornet et al., 1981;

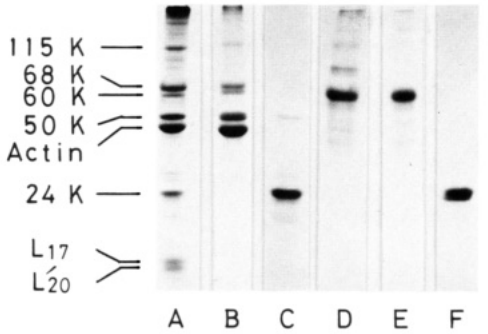

FIGURE 6: $NaDodSO_4$ gel electrophoretic patterns of various elution peaks. (A) Mixture obtained after cross-linking the acto–tryptic gizzard HMM with EDC. (B and C) Samples obtained by gel filtration on a Sephadex G-100 column (Figure 5A, peaks I and II, respectively). (D) The elution peak obtained by chromatography on a CM-52 column (hatched area in Figure 5B). (E) 60K cross-linked homodimer of the 24K fragment isolated by chromatography on a Mono S column (Figure 5C). (F) 24K fragment of the HMM heavy chain isolated by chromatography on a CM-52 column (Figure 5D).

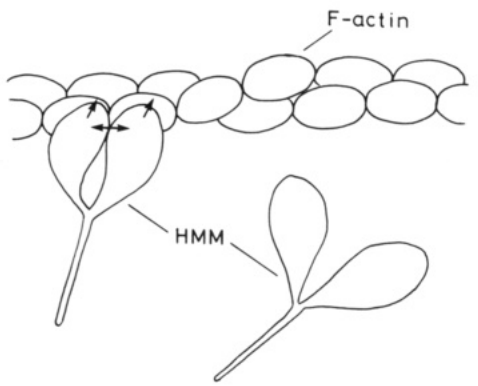

FIGURE 7: Schematic illustration of two types of carbodiimide-catalyzed covalent cross-linking between F-actin and gizzard HMM during rigor complex formation. Cross-linking occurs between the two HMM heads (double-headed arrow), as well as between F-actin and each HMM head (arrows). As illustrated, no head-to-head cross-linking occurs in HMM molecules that are not bound to F-actin.

Table I: Amino Acid Compositions of 24K and 60K Peptides[a]

	24K	60K		24K	60K
Asp	21.32 (21)	20.54	Tyr	8.73 (9)	8.77
Glu	25.68 (25)	26.61	Val	12.74 (14)	12.21
cmCys[b]	nd[c] (3)	nd	Met	5.03 (5)	5.88
Ser	14.83 (16)	14.76	Ile	10.45 (11)	9.30
Gly	10.23 (10)	10.71	Leu	16.02 (16)	17.43
His	4.39 (5)	4.26	Phe	7.25 (7)	7.10
Arg	5.50 (5)	5.92	Trp	nd (2)	nd
Thr	8.52 (8)	8.63	Lys	24.67 (24)	23.10
Ala	13.26 (13)	13.22	total no. of amino acids	203	
Pro	9.38 (9)	9.55			

[a] The number of amino acid residues in parentheses are values from sequence data (Maita et al., 1987). [b] *S*-(Carboxymethyl)cysteine. [c] nd = not determined.

Sutoh, 1982, 1983), no such contacts were revealed with acto–gizzard S-1 (Marianne-Pépin et al., 1985) or acto–gizzard HMM (present study).

On the basis of its apparent molecular weight and amino acid composition, we suggest that the 60K peptide is the cross-linked complex of the two NH_2-terminal 24K fragments of the HMM heavy chain. We further suggest that when gizzard HMM attaches to F-actin under physiological conditions, the two heads of a myosin molecule are in contact with each other via the 24K segment region (double-headed arrow in Figure 7). The highly efficient production of the 60K cross-linked peptide suggests that the complex formed between the two HMM heads attached to F-actin is quite stable. Furthermore, we did not observe head-to-head cross-linking when gizzard HMM alone was treated with EDC. Thus, we suggest that the association of the two HMM heads occurs only when HMM is bound to F-actin.

Covalent cross-linking between the two myosin heads was not observed in previous studies using either skeletal muscle S-1 or gizzard S-1. There are two plausible reasons that can explain the absence of EDC cross-linking between the two myosin heads in these earlier works: (1) Cross-linking of the two S-1 molecules attached to F-actin may occur with much less efficiency than the cross-linking of the two heads of a HMM molecule bound to F-actin. (2) In previous studies, the cross-linking reaction was initiated by mixing EDC-activated F-actin with skeletal muscle S-1 or gizzard S-1, whereas in this study, we initially mixed F-actin with HMM and then initiated cross-linking by the addition of EDC.

Gizzard S-1, with or without the regulatory light chain, was fully activatable by actin (Nath et al., 1982). Covalent attachment of F-actin to gizzard S-1 lacking the regulatory light chain or to its fragmented derivatives using EDC was also shown to increase the Mg-ATPase activity of S-1 (Marianne-Pépin et al., 1985). The authors suggested that when S-1 is covalently linked to actin, the increase in Mg-ATPase activity occurs because the actin-binding site on S-1 is irreversibly occupied by actin during cross-linking. However, when the regulatory light chains of gizzard HMM were cleaved with trypsin, its ATPase activity was no longer activatable by actin (Onishi & Watanabe, 1979; Seidel, 1980). When we combined F-actin with HMM lacking regulatory light chains and then initiated cross-linking by the addition of EDC, we observed an accelerated Mg-ATPase activity of the acto–HMM complex. Thus, during the covalent cross-linking of HMM to actin, not only is the actin-binding site on HMM irreversibly occupied by actin, but also the conformation of HMM may change from an inactive form to an active form. The covalently linked acto–gizzard HMM complex provides valuable material for investigating the actin activation of myosin Mg-ATPase activity.

Our observations also indicate that the Mg-ATPase activity of the covalently linked acto–HMM complex significantly decreased when the 68K fragment of HMM was modified by IAEDANS (see Figure 4, parts III and IV). Only one modified cysteine residue was present in this fragment, and it was identified as SH_1 (Onishi et al., 1986), on the basis of its sequence homology with rabbit skeletal muscle myosin (Elzinga & Collins, 1977). Therefore, we suggest that the decrease in acto–HMM Mg-ATPase activity is due to blocking of the SH_1 residue of gizzard myosin. Previous studies have shown that blocking the gizzard myosin SH_1 residue depressed the Mg-ATPase activity of phosphorylated actomyosin, although the phosphorylation was unaffected in the SH_1-blocked myosin (Onishi, 1985; Nath et al., 1986). It is thus likely that the SH_1 region of gizzard myosin, which is closely related to the Mg-ATPase site (Onishi & Watanabe, 1985), is required for high actomyosin ATPase activity.

In this study, we have demonstrated that a head-to-head interaction occurs in HMM when gizzard HMM binds to F-actin, forming a rigor complex. A homodimer of NH_2-terminal 24K fragments was generated during EDC-induced cross-linking. The homodimer has been characterized, and our results are presented in the following paper in this issue (Onishi et al., 1989). We also showed that the Mg-ATPase activity of gizzard HMM lacking regulatory light chains is

enhanced by the covalent cross-linking of HMM to actin.

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REFERENCES

Bâlint, M., Wolf, I., Tarcsafalvi, A., Gergely, J. & Srêter, F. A. (1978) *Arch. Biochem. Biophys. 190*, 793–799.

Ballentine, R., & Gregg, J. R. (1947) *Anal. Chem. 19*, 281–283.

Crestfield, A. M., Moore, S., & Stein, W. H. (1963) *J. Biol. Chem. 238*, 622–627.

Elzinga, M., & Collins, J. H. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 4281–4284.

Gornall, A. G., Bardawill, C. J., & David, M. M. (1949) *J. Biol. Chem. 177*, 751–766.

Ikebe, M., Aiba, T., Onishi, H., & Watanabe, S. (1978) *J. Biochem. 83*, 1643–1655.

Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.

Lowey, S., Slayter, H. S., Weeds, A. G., & Baker, H. (1969) *J. Mol. Biol. 42*, 1–29.

Maita, T., Onishi, H., Yajima, E., & Matsuda, G. (1987) *J. Biochem. 102*, 133–145.

Marianne-Pêpin, T., Mornet, D., Bertrand, R., Labbê, J.-P., & Kassab, R. (1985) *Biochemistry 24*, 3024–3029.

Mornet, D., Bertrand, R., Pantel, P., Audemard, E., & Kassab, R. (1981) *Nature (London) 292*, 301–306.

Nath, N., Chandra, T. S., Suzuki, H., Carlos, A., & Seidel, J. C. (1982) *Biophys. J. 37*, 47a.

Nath, N., Nag, S., & Seidel, J. C. (1986) *Biochemistry 25*, 6169–6176.

Onishi, H. (1985) *J. Biochem. 98*, 81–86.

Onishi, H., & Watanabe, S. (1979) *J. Biochem. 85*, 457–472.

Onishi, H., & Watanabe, S. (1985) *Adv. Biophys. 19*, 21–42.

Onishi, H., Maita, T., Miyanishi, T., Watanabe, S., & Matsuda, G. (1986) *J. Biochem. 100*, 1433–1447.

Onishi, H., Maita, T., Matsuda, G., & Fujiwara, K. (1989) *Biochemistry* (following paper in this issue).

Pollard, T. D., & Korn, E. D. (1973) *J. Biol. Chem. 248*, 4682–4690.

Seidel, J. C. (1980) *J. Biol. Chem. 255*, 4355–4361.

Spudich, J. A., & Watt, S. (1971) *J. Biol. Chem. 246*, 4866–4871.

Sutoh, K. (1982) *Biochemistry 21*, 4800–4804.

Sutoh, K. (1983) *Biochemistry 22*, 1579–1585.

Yamamoto, K., Sekine, T., & Kanaoka, Y. (1977) *Anal. Biochem. 79*, 83–94.

Yanagida, T., Nakase, M., Nishiyama, K., & Oosawa, F. (1984) *Nature (London) 307*, 58–60.

Yanagisawa, M., Hamada, Y., Katsuragawa, Y., Imamura, M., Mikawa, T., & Masaki, T. (1987) *J. Mol. Biol. 198*, 143–157.

Yano-Toyoshima, Y., Kron, S. J., McNally, E. M., Neibling, K. R., Toyoshima, C., & Spudich, J. A. (1987) *Nature (London) 328*, 536–539.

Youngburg, G. E., & Youngburg, M. V. (1930) *J. Lab. Clin. Med. 16*, 158–166.