Interaction between the Heavy and the Regulatory Light Chains in Smooth Muscle Myosin Subfragment 1†

Hirofumi Onishi,*,‡ Tetsuo Maita,§ Genji Matsuda,§ and Keigi Fujiwara‡

Department of Structural Analysis, National Cardiovascular Center Research Institute, Fujishiro-dai, Suita, Osaka 565, Japan, and Department of Biochemistry, Nagasaki University School of Medicine, Nagasaki, Nagasaki 852, Japan

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ABSTRACT: The interaction between the heavy and the regulatory light chains within chicken gizzard myosin heads was investigated by using a zero-length chemical cross-linker, 1-ethyl-3-[3-(dimethylamino)-propyl]carbodiimide (EDC). The chicken gizzard subfragment 1 (S-1) used was treated with papain so that the heavy chain was partly cleaved into the NH_2-terminal 72K and the COOH-terminal 24K fragments and the regulatory light chain into the 16K fragment. S-1 was reacted with EDC either alone or in the presence of ATP or F-actin. In all cases, the 16K fragment of the regulatory light chain formed a covalent cross-link with the 24K heavy chain fragment but not with the 72K fragment. The 38K cross-linked peptide, which was the product of cross-linking between the 16K light chain and the 24K heavy chain fragments, was isolated and further cleaved with cyanogen bromide and arginylendopeptidase. Smaller cross-linked peptides were purified by reverse-phase HPLC and then characterized by amino acid analysis and sequencing. The results indicated that cross-linking occurred between Lys-845 in the heavy chain and Asp-168, Asp-170, or Asp-171 in the regulatory light chain. The position of the cross-linked lysine was only three amino acid residues away from the invariant proline residue mapped as the S-1-rod hinge by McLachlan and Karn [McLachlan, A. D., & Karn, J. (1982) *Nature (London) 299*, 226–231]. We propose that the COOH-terminal region of the regulatory light chain is located in the neck region of myosin and that this region and the phosphorylation site of the regulatory light chain together may play a role in the phosphorylation-induced conformational change of gizzard myosin.

The force-generating reaction of muscle contraction is controlled by regulatory proteins. In vertebrate smooth muscle and nonmuscle cells, the myosin molecule itself contains the regulatory subunits (Sobieszek, 1977; Aksoy et al., 1976; Chacko et al., 1977; Ikebe et al., 1977; Scholey et al., 1980). Two types of small subunits known as the regulatory (20K) and the essential (17K) light chains are located in the globular head portion of myosin. When regulatory light chains are not phosphorylated, there is no actin-activated ATPase activity of myosin. Ca^{2+} ions activate myosin light chain kinase which phosphorylates the regulatory light chains. The actin-activated ATPase activity is switched on as a result of phosphorylation. In order to understand how regulatory light chains control the interaction of myosin heads with F-actin, the precise location of the light chains within myosin heads must be determined.

Regulatory light chains of scallop myosin can be reversibly dissociated by EDTA. Vibert and Craig (1982) and Flicker et al. (1983) have demonstrated increased electron density due to regulatory light chains in the neck region of scallop myosin heads. Since this finding, many electron microscopic and biochemical studies have shown that regulatory light chains are associated with the COOH-terminal region of the S-1[1] heavy chain, which is localized in the neck region (Winkelmann et al., 1983; Szentkiralyi, 1984; Sellers & Harvey, 1984; Mitchell et al., 1986, 1989). Little is known, however, about the identity of particular amino acid residues involved in the regulatory light chain binding to the myosin heavy chain. In the present study, we have used a zero-length cross-linking technique to demonstrate a possible site for direct interaction between the heavy and the regulatory light chains within smooth muscle myosin heads. This is the first report to identify the amino acid residues for the interaction between the heavy and the light chains.

Materials and Methods

Protein Preparations. Chicken gizzard myosin was prepared as described in our previous report (Ikebe et al., 1978). Rabbit skeletal muscle F-actin was prepared by the method of Spudich and Watt (1971).

Gizzard S-1 was prepared by digesting myosin with papain according to Marianne-Pépin et al. (1983) with a slight modification. Briefly, gizzard myosin (2 mg/mL) in 0.6 M KCl, 10 mM $MgCl_2$, and 20 mM Tris-HCl (pH 7.5) was digested with papain (weight ratio: 1:100 protease/myosin) at 20 °C for 60 min, and digestion was terminated by adding 10 mM iodoacetic acid. S-1 was then purified by chromatography on a DEAE-cellulose column. In our S-1 preparations, the heavy chain was partly cleaved into 72K and 24K segments. Although the S-1 heavy chain and its 72K and 24K segments were further degraded at the NH_2- or COOH-terminal region (Onishi & Watanabe, 1984), these products represented only the minor components in the preparation. The regulatory light chain was also cleaved at the NH_2-terminal region to form the 16K fragment.

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* Address correspondence to this author.

‡ National Cardiovascular Center Research Institute.

§ Nagasaki University School of Medicine.

[1] Abbreviations: EDC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide; S-1, subfragment 1; DTT, dithiothreitol; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; $NaDodSO_4$, sodium dodecyl sulfate; HPLC, high-performance liquid chromatography; PTH, phenylthiohydantoin.

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Cross-Linking of S-1 or Acto–S-1. The cross-linking reaction with EDC was carried out essentially as described in our previous report (Onishi et al., 1989). Briefly, gizzard S-1 (0.7 mg/mL) in 0.1 M KCl, 2 mM $MgCl_2$, and 10 mM imidazole hydrochloride (pH 7.0) was incubated with freshly prepared 5 mM EDC for 1 or 2 h at 24 °C, and the reaction was terminated by adding 30 mM 2-mercaptoethanol. For the EDC cross-linking reaction of acto–S-1, gizzard S-1 (0.7 mg/mL) was mixed with skeletal muscle F-actin, with a molar ratio of actin/S-1 = 1, and cross-linked as described.

Gel Electrophoresis and Immunoblotting. After cross-linking for 1 or 2 h, an aliquot from the reaction mixture was submitted to electrophoresis in the presence of $NaDodSO_4$ (Laemmli, 1970), using polyacrylamide slab gels containing 11% acrylamide and 0.3% bisacrylamide. Gels were stained with Coomassie brilliant blue. Apparent molecular weights of cross-linked peptides were determined from their electrophoretic mobilities using following markers: myosin heavy chain (200K), β-galactosidase (116K), phosphorylase B (93K), bovine serum albumin (66K), and ovalbumin (45K).

For immunoblots, peptide bands were electrophoretically transferred to a Durapore membrane (Millipore Co., Bedford, MA) (Towbin et al., 1979). The membrane was immunostained with affinity-purified polyclonal antibodies against rabbit skeletal actin followed by peroxidase-conjugated goat anti-rabbit IgG (Cooper Biomedical Inc., West Chester, PA). The reacting peptide bands were visualized by using immunostaining kit IS-50B purchased from Konica Co. (Tokyo, Japan).

S-Carboxymethylation. The cross-linked products of gizzard S-1 were collected by salting out with ammonium sulfate at 67% saturation and then were dissolved in a denaturating solution containing 6 M guanidine hydrochloride, 5 mM EDTA, and 0.5 M Tris-HCl (pH 8.0). Under a nitrogen barrier, the sample was reduced with 100 mM 2-mercaptoethanol for 8 h at 40 °C. A freshly prepared solution of iodoacetic acid, in an amount equal to 2-mercaptoethanol on a molar basis, was added to the reaction mixture, and the incubation was continued for 1 h (Crestfield et al., 1963).

Preparation of Cross-Linked Peptides. The *S*-carboxymethylated S-1 sample was submitted to gel filtration on a Sephacryl S-300HR column equilibrated with a solution containing 5 M guanidine hydrochloride, 20 mM Tris-HCl (pH 8.0), and 1 mM EDTA. Three fractions, each of which consisted mainly of the 16K light chain fragment, the 24K heavy chain fragment, or the 38K cross-linked complex, were further purified by reverse-phase HPLC using a Zorbax Pro 10/300 column (0.46 × 25 cm or 2 × 25 cm, Du Pont, MA). Solvent A was 0.1% trifluoroacetic acid in acetonitrile/water (1:19 v/v), and solvent B was 0.09% trifluoroacetic acid in acetonitrile/water (9:1 v/v).

For the preparation of smaller cross-linked peptides, the 38K cross-linked complex was dissolved in 0.3 mL of 70% formic acid containing 0.12 g of cyanogen bromide (CNBr) and incubated at room temperature for 24 h. Separation of CNBr peptides was accomplished by the same reverse-phase HPLC except that a Wakosil C_8 column (0.6 × 25 cm, Wako Chemical Industries, Osaka, Japan) was used instead of a Zorbax column. For arginylendopeptidase digestion, the cross-linked fraction isolated from the CNBr digest was dissolved in 0.25 mL of 50 mM Tris-HCl buffer (pH 8.6). To this was added 10 μL of a stock solution of 1 mg/mL arginylendopeptidase (Takara Shuzo, Kyoto, Japan). Digestion was allowed to proceed at 37 °C for 6 h. Arginylendopeptidase peptides were subjected to reverse-phase HPLC on a Cosmosil C_{18} column (0.46 × 25 cm, Nakarai tesque, Kyoto, Japan). Solvent A used for this chromatography was 0.1% trifluoroacetic acid in water, and solvent B was 0.09% trifluoroacetic acid in acetonitrile/water (9:1 v/v). The cross-linked product enriched in one fraction was further purified by HPLC on a Vydac diphenyl column (0.46 × 25 cm, The Separations Group, Hesperia, CA) with the same solvent system as that used for the C_{18} column. Control samples including the uncross-linked 16K light chain fragment and the uncross-linked 24K heavy chain fragment were also digested and analyzed by HPLC performed under the same conditions used for the 38K cross-linked complex. Absorbance was monitored at 210 nm.

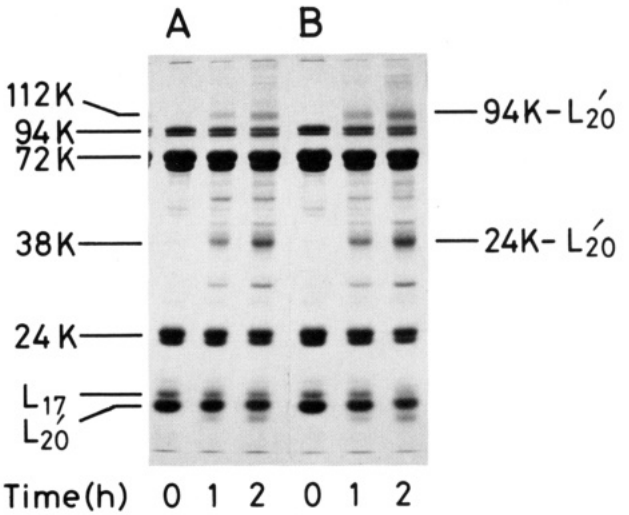

FIGURE 1: $NaDodSO_4$ gel electrophoretic patterns of the EDC cross-linked products of gizzard S-1. Gizzard S-1 (0.7 mg/mL) was incubated with 5 mM EDC as specified under Materials and Methods. The cross-linking reaction was performed in the absence (A) or presence (B) of 2 mM ATP. At 0 h (i.e., not cross-linked) and at 1 or 2 h following cross-linking (indicated at the bottom of each lane), aliquots were withdrawn and mixed with 2-mercaptoethanol to terminate the reaction. Protein samples (7 μg of S-1) were submitted to gel electrophoresis in the presence of $NaDodSO_4$. The M_r of the intact S-1 heavy chain is 94K, while 72K and 24K are papain fragments of the heavy chain. L_{17} and L_{20}' indicate the essential light chain and the 16K fragment of the regulatory light chain, respectively. The two cross-linked species, 112K (94K–L_{20}') and 38K (24K–L_{20}'), are indicated. These species are produced in either the presence or the absence of ATP.

Amino Acid Compositions and Sequence Analysis. Peptide samples were hydrolyzed, derivatized with phenylthiocyanate, and analyzed by reverse-phase HPLC as described in our previous report (Onishi et al., 1989). 3-Bromotyrosine used as a control was prepared by bromination of tyrosine (Welinder, 1972). Peptide sequencing was carried out by using an Applied Biosystems model 477A gas-phase protein sequencer, equipped with an on-line model 120A PTH amino acid analyzer.

Mass Spectrometry. Mass spectra were obtained with a double-focusing mass spectrometer (JEOL JMS-HX100) equipped with a fast atom bombardment ion source and a data processor (JEOL JMA-DA5000), as described by Takao et al. (1984). Mass assignment was made using a mixture of CsI and KI as a mass reference. A sample solution containing 0.5 μg of peptides was loaded on a stainless steel plate and mixed with glycerol on the plate.

RESULTS

EDC Cross-Linking of Gizzard S-1. When gizzard S-1 was incubated with EDC in the presence or absence of 2 mM ATP, distinct cross-linked products were formed. $NaDodSO_4$ gel

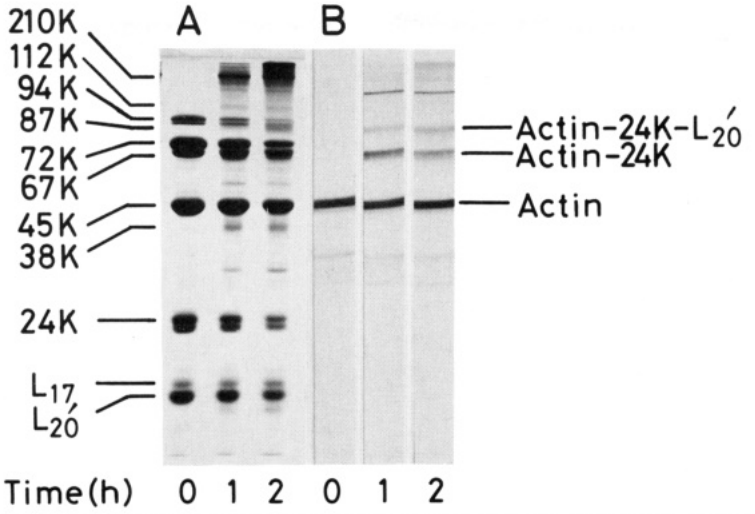

FIGURE 2: $NaDodSO_4$ gel electrophoretic patterns of the EDC cross-linked products of acto–gizzard S-1. Gizzard S-1 (0.7 mg/mL) in a rigor complex with F-actin (0.25 mg/mL) was incubated with 5 mM EDC as specified under Materials and Methods for 0 (not cross-linked), 1, or 2 h as indicated at the bottom of each lane. Polypeptide bands were stained with Coomassie blue (A). Bands containing actin were identified by staining electroblotted bands with the antibody against actin (B). Cross-linked products of 67K (actin–24K) and 87K (actin–24K–L_{20}') as well as actin are immunostained. The immunostained band of approximately 150K is not the product of the actin–S-1 cross-linking reaction, as it appeared when F-actin alone was treated with EDC.

electrophoretic patterns of aliquots from EDC reaction mixture without ATP shown in panel A of Figure 1 indicate that two broad protein bands appeared during treatment with EDC for 1 or 2 h. Apparent molecular weights (M_r's) of the two bands were approximately 112K and 38K. As described under Materials and Methods, the 94K heavy chain and its NH_2-terminal 72K and COOH-terminal 24K fragments were present in our S-1 preparations. The bands immediately below the 94K, 72K, and 24K bands represent further digested fragments of the S-1 heavy chain and its NH_2-terminal and COOH-terminal fragments, respectively (Onishi & Watanabe, 1984). M_r's 112K and 38K were larger by approximately 16K than the M_r values for the S-1 heavy chain and its COOH-terminal fragment, respectively. Since M_r of the regulatory light chain fragment is approximately 16K, we tentatively conclude that the 112K polypeptide is the cross-linked product between the S-1 heavy chain and the 16K light chain fragment and that the 38K polypeptide is the cross-linked product between the 24K heavy chain fragment and the 16K light chain fragment. In addition to cross-linked peptide bands, several new bands appeared in the M_r range between 24K and 72K as the result of EDC treatment. HPLC elution profiles of the CNBr digests of these polypeptides indicated that neither the 24K heavy chain fragment nor the 16K light chain fragment was present as components (data not shown). These bands are presumably degraded products of the NH_2-terminal 72K fragment of the S-1 heavy chain, not cross-linked products. Panel B in Figure 1 shows the gel patterns of aliquots cross-linked with EDC for 0, 1, or 2 h in the presence of ATP. These patterns were quite similar to those shown in Figure 1A, indicating that the ATP-induced conformational change in S-1 does not affect the cross-linking reaction.

S-1 binds to F-actin, thus forming the rigor complex. The gel electrophoretic patterns in panel A of Figure 2 show that five cross-linked polypeptides of M_r's 210K, 112K, 87K, 67K, and 38K are produced during EDC-catalyzed reaction of the rigor complex for 1 or 2 h. As already described, the 112K and 38K species are two intramolecular cross-linked products in S-1 and do not involve actin. As an attempt to detect actin-containing bands, we used affinity-purified antiactin. EDC-catalyzed reaction mixtures of the rigor complex were electrophoresed and then blotted on to a Durapore membrane. The immunostaining of the membrane with antibodies against rabbit actin was carried out, and the results are shown in panel B of Figure 2. Antiactin stained the 87K and 67K bands but not the 210K band. Our previous study using fluorescent F-actin (Onishi et al., 1989) has already identified the 67K species as the cross-linked product between actin and the COOH-terminal 24K fragment of the S-1 heavy chain. Since M_r 87K was larger by approximately 16K than the M_r value for the actin–24K cross-linked complex, we tentatively concluded that the 87K species was a complex of three peptides: actin, the 24K heavy chain fragment, and the 16K light chain fragment. The 210K cross-linked complex must be composed of only S-1 components, because the 210K band did not contain actin. We suggest that cross-linking between heavy chain NH_2-terminal regions of two S-1's is involved in the production of this high M_r species, because we have already shown that EDC cross-links two heavy chains of gizzard heavy meromyosin attached to F-actin (Onishi et al., 1989). The band seen in lanes 1 and 2, but not in lane 0, of Figure 2B at M_r approximate 150K is not a product of the actin–S-1 cross-linking reaction, as it appeared when F-actin alone was treated with EDC under the same conditions as used for cross-linking the rigor complex (data not shown). As previously described (Onishi et al., 1989), trace amounts of α-actinin were present in our F-actin preparation. It is likely, therefore, that the 150K species is the cross-linked complex of actin and α-actinin.

Isolation of the 38K Cross-Linked Product. EDC cross-linked gizzard S-1 was denatured with 6 M guanidine hydrochloride and *S*-carboxymethylated (see Materials and Methods). The *S*-carboxymethylated sample was subjected to gel filtration on a Sephacryl S-300HR column. The elution profile illustrated in Figure 3A shows four major peaks. Peptides present in various fractions were analyzed by $NaDodSO_4$ gel electrophoresis. The 38K cross-linked complex (Figure 4, lane A) was present in fractions 44–46, the 24K heavy chain fragment (Figure 4, lane B) was present in fractions 49–51, and two types of light chains (Figure 4, lane C) were present in fractions 52–54.

The two types of light chains in fraction 53 were separated by reverse-phase HPLC on a Zorbax Pro 10/300 column. The HPLC elution profile is shown in Figure 3B and reveals two

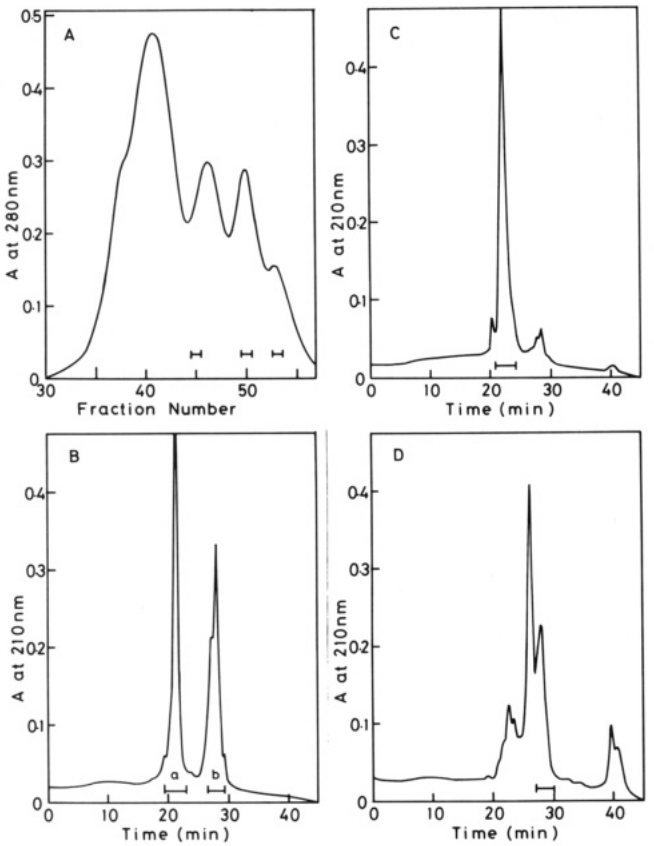

FIGURE 3: Separation of two light chains, the 24K heavy chain fragment, and the 38K cross-linked complex from the EDC-catalyzed cross-linked products. (A) *S*-Carboxymethylated derivatives of cross-linked products were applied to a Sephacryl S-300HR column (2.6 × 100 cm) that had been equilibrated with 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 0.7 mL/min. Fractions 53, 50, and 45 (bracketed in Figure 3A) were separately rechromatographed on a reverse-phase HPLC column (0.46 × 25 cm) of Zorbax Pro 10/300 (panels B, C, and D, respectively). Peptides were eluted with two successive linear gradients of acetonitrile. The concentration gradient was from 20 to 40% solvent B between 0 and 5 min and from 40 to 70% solvent B between 5 and 45 min. The flow rate was 1 mL/min.

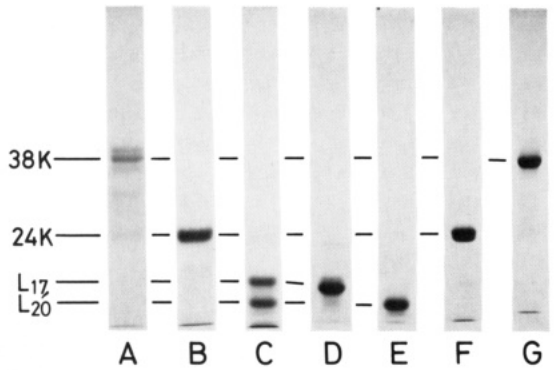

FIGURE 4: $NaDodSO_4$ gel electrophoretic patterns of various peptide samples. (A, B, and C) Samples obtained after gel filtration on a Sephacryl S-300HR column (Figure 3A, fractions 45, 50, and 53, respectively). (D) The essential light chain (L_{17}) (Figure 3B, peak a). (E) The 16K fragment (L_{20}') of the regulatory light chain (Figure 3B, peak b). (F) The COOH-terminal 24K fragment of the heavy chain (Figure 3C). (G) The 38K cross-linked complex (Figure 3D).

peaks at 21.8 and 28.1 min (denoted as a and b, respectively). Analyses of these fractions by $NaDodSO_4$ gel electrophoresis indicated that peak a contains the 17K peptide (Figure 4, lane D) and peak b the 16K peptide (Figure 4, lane E). Edman degradation of the 17K peptide showed no PTH-amino acids,

Table I: Designation of CNBr Peptides of the Regulatory Light Chain and the 24K Heavy Chain Fragment[a]

regulatory light chain		24K heavy chain fragment	
CNBr peptides	residues included	CNBr peptides	residues included
CB1′	17–24	CN1	642–654
CB1″	20–24	CN1′	647–654
CB2	25–39	CN2	655–670
CB3	40–56	CN3	671–746
CB4	57–60	CN4	747–755
CB5	61–72	CN5	756–821
CB7	74–84	CN6	822–854
CB8	85–88		
CB9	89–129		
CB10	130–141		
CB11	142–171		

[a] CB designates the CNBr peptides of the regulatory light chain (Maita et al., 1981), and CN designates the CNBr peptides of the 24K heavy chain fragment (Onishi et al., 1986). The heavy chain numbering system is offset by one residue from that based on the deduced amino acid sequence from Yanagisawa et al. (1987), because the NH_2-terminal Met could not be found in the sequence of the NH_2-terminal 24K fragment of gizzard myosin (Maita et al., 1987).

indicating that the NH_2 terminus is blocked. Judging from its molecular weight, we identified this polypeptide to be the essential light chain. On the other hand, Edman degradation of the 16K peptide produced two PTH-amino acids at each cycle. After five cycles of degradation, we were able to construct two five-residue sequences, ATSNV and NVFAM, which corresponded to partial amino acid sequences of the regulatory light chain. The first pentamer was identical to the regulatory light chain sequence from the 17th to the 21st amino acid residues, and the second from the 20th to the 24th residues. Therefore, we conclude that peak b contains two truncated COOH-terminal fragments of the regulatory light chain.

The 24K heavy chain fragment-rich fraction 50 in Figure 3A was further purified by reverse-phase HPLC (Figure 3C), and only one peak eluted at 21.9 min. The pure 24K fragment was obtained by this chromatography (Figure 4, lane F). The 38K cross-linked complex-rich fraction 45 in Figure 3A was further purified by reverse-phase HPLC. The elution profile (Figure 3D) shows that there are two major peaks at 26.3 and 28.2 min. Fractions eluted between 27 and 30 min were pooled and used as the purified cross-linked complex (Figure 4, lane G).

Identification of Cross-Linked Sites. Figure 5, panels A and B, show HPLC elution profiles of CNBr digests of uncross-linked 16K light chain and 24K heavy chain fragments, respectively. Sequence analyses of various peaks permitted the identification of all the expected CNBr peptides from these uncross-linked fragments (see Table I and the legend to Figure 5). Peptides within peaks 5 and 6 in Figure 5A had YR-FAPIDKK as the first nine amino acid sequence, suggesting that both peaks contain the peptide CB11 of the regulatory light chain (RLC–CB11). The difference between these two CB11 peptides will be discussed later. The first five amino acids in the sequence of the peptide within peak 3 in Figure 5B was KVIQR, indicating that this peak contains the peptide CN6 of the 24K heavy chain fragment (24K–CN6).

The HPLC elution profile of the CNBr-digested 38K cross-linked complex is shown in Figure 5C. Most of the peaks in this profile have corresponding peaks in either Figure 5A or Figure 5B. However, a broad peak X which coeluted with peak 3 in Figure 5B is unique to Figure 5C, and the two RLC–CB11-containing peaks (peaks 5 and 6 in Figure 5A) are missing in Figure 5C. These results suggest that the 38K

Table II: Amino Acid Composition of the RLC–CB11 Arginylendopeptidase Fragments Separated by Reverse-Phase HPLC[a]

	column peaks							
	1–3	4–5	6	7	8	9	8*	9*
Asp	2.91 (3)		0.98 (1)		2.58 (3)	2.14 (2)	2.68 (3)	2.02 (2)
Glu			1.00 (1)		1.89 (2)	1.09 (1)	2.09 (2)	1.16 (1)
Gly	1.00 (1)				1.08 (1)	1.01 (1)	1.06 (1)	0.98 (1)
His	0.95 (1)							
Arg		0.98 (1)			1.05 (1)	1.10 (1)	0.88 (1)	0.95 (1)
Thr					1.13 (1)	0.84 (1)	0.93 (1)	1.00 (1)
Ala	1.01 (1)		1.03 (1)		1.01 (1)		1.05 (1)	
Pro			1.03 (1)		1.12 (1)		1.01 (1)	
Tyr		1.02 (1)			1.06 (1)	0.94 (1)	(1)	(1)
Val					1.05 (1)	1.04 (1)	1.07 (1)	1.01 (1)
Ile			1.06 (1)	0.99 (1)	1.11 (1)		2.01 (1)	1.02 (0)
Leu				1.00 (1)				
Phe					2.06 (2)	1.84 (2)	2.18 (2)	1.87 (2)
Lys	2.13 (2)		1.90 (2)	1.01 (1)	1.85 (2)		2.04 (2)	
identified sequence fragment	164–171	142–143	144–150	161–163	144–160	151–160	144–160	151–160
yield (%)	48	51	15	59	35	19	37	19

[a] The numbers of amino acid residues in parentheses are expected values determined from the sequence data (Maita et al., 1981).

Table III: Amino Acid Compositions of the 24K–CN6 Arginylendopeptidase Fragments Separated by Reverse-Phase HPLC[a]

	column peaks								
	1–2	3	4	5	6	7	8–13	14	15
Asp				1.19 (1)		1.61 (1.5)	1.01 (1)		
Glu		1.04 (1)			1.14 (1)	0.57 (0.5)	1.00 (1)	1.18 (1)	0.97 (1)
cmCys[b]				0.60 (1)		0.76 (1.0)			
Arg	1.00 (1)	0.96 (1)			1.07 (1)	1.39 (1.5)	0.99 (1)		0.97 (1)
Thr			0.90 (1)		0.98 (1)			1.01 (1)	1.85 (2)
Ala				1.86 (2)		1.97 (2.0)			
Pro					1.02 (1)			1.04 (1)	1.10 (1)
Tyr				0.98 (1)		0.97 (1.0)			
Val		0.68 (1)			1.91 (2)			0.99 (1)	1.92 (2)
Ile		0.76 (1)							
Leu	1.00 (1)		1.05 (1)	1.02 (1)	2.22 (2)	2.07 (2.0)		3.03 (3)	3.08 (3)
Phe			1.00 (1)					1.01 (1)	1.12 (1)
Trp						nd (3.0)[c]	nd (3)[c]		
Lys		1.00 (1)	1.04 (1)	0.94 (1)	1.03 (1)	1.00 (1.0)		1.91 (2)	2.00 (2)
identified sequence fragment	834–835	822–826	842–845	827–833	846–854	827–835 and 836–841	836–841	842–851	842–854
yield (%)	28	56	16	38	13	29 and 15	28	11	35

[a] The numbers of amino acid residues in parentheses are expected values determined from the sequence data (Onishi et al., 1986). [b] *S*-(Carboxymethyl)cysteine. [c] Not determined.

cross-linked complex contains 16K light chain and 24K heavy chain fragments as its components. Indeed, a sequence analysis of peak X revealed that the amino acid residues present in RLC–CB11 and 24K–CN6 were found in this peak. Since RLC–CB11 normally elutes much earlier than peak X, its presence in the late-eluting peak X indicates that it is cross-linked to 24K–CN6.

In order to identify EDC cross-linking sites within both RLC–CB11 and 24K–CN6 sequences, the two RLC–CB11 types (peaks 5 and 6 in Figure 5A), the 24K–CN6 (peak 3 in Figure 5B), and their cross-linked complex (peak X in Figure 5C) were separately digested by arginylendopeptidase under the same conditions, and peptide fragments were analyzed by reverse-phase HPLC. HPLC elution profiles of the arginylendopeptidase digests of the two RLC–CB11 types show nine major peaks (Figure 6A,B). As shown in Table II, the amino acid composition of the numbered peaks except for peaks 8* and 9* was similar to those of expected arginylendopeptidase fragments from RLC–CB11. Peaks 1–3, 4–5, 6, 7, 8, and 9 were thus identified to be regulatory light chain fragments consisting of residues 164–171, 142–143, 144–150, 161–163, 144–160, and 151–160, respectively. Although elution profiles in Figure 6, panels A and B are similar, elution times of the two late-eluting peaks 8* and 9* were different from those of peaks 8 and 9, respectively. Amino acid analyses of peaks 8* and 9* indicated that tyrosine and isoleucine contents were different from the theoretical values calculated from regulatory light chain sequences 144–160 and 151–160, respectively. These two sequences contain only one tyrosine at position 155. Therefore, this residue must be modified or substituted by isoleucine. The HPLC elution profile of the arginylendopeptidase digested 24K–CN6 shows 15 major peaks (Figure 6C). As shown in Table III, the amino acid composition of peaks 1–2, 3, 4, 5, 6, 8–13, 14, and 15 was similar to that of expected heavy chain fragments consisting of residues 834–835, 822–826, 842–845, 827–833, 846–854, 836–841, 842–851, and 842–854, respectively. The amino acid composition of peak 7 could be obtained from a mixture of peptides consisting of residues 827–835 and 836–841. These analyses enabled us to identify all of the amino acid residues in both RLC–CB11 and 24K–CN6 fragments.

The HPLC elution profile of the arginylendopeptidase fragments obtained from the cross-linked complex (peak X) is shown in Figure 6D. The three early-eluting peaks containing residues 164–171 of the regulatory light chain (peaks 1–3 in Figure 6A,B) were not present in the arginylendopeptidase sample of peak X. The three peaks containing residues 842–845, 846–854, and 842–854 of the heavy chain (peaks 4, 6, and 15 in Figure 6C) were also greatly reduced. These results suggest that these arginylendopeptidase peptides

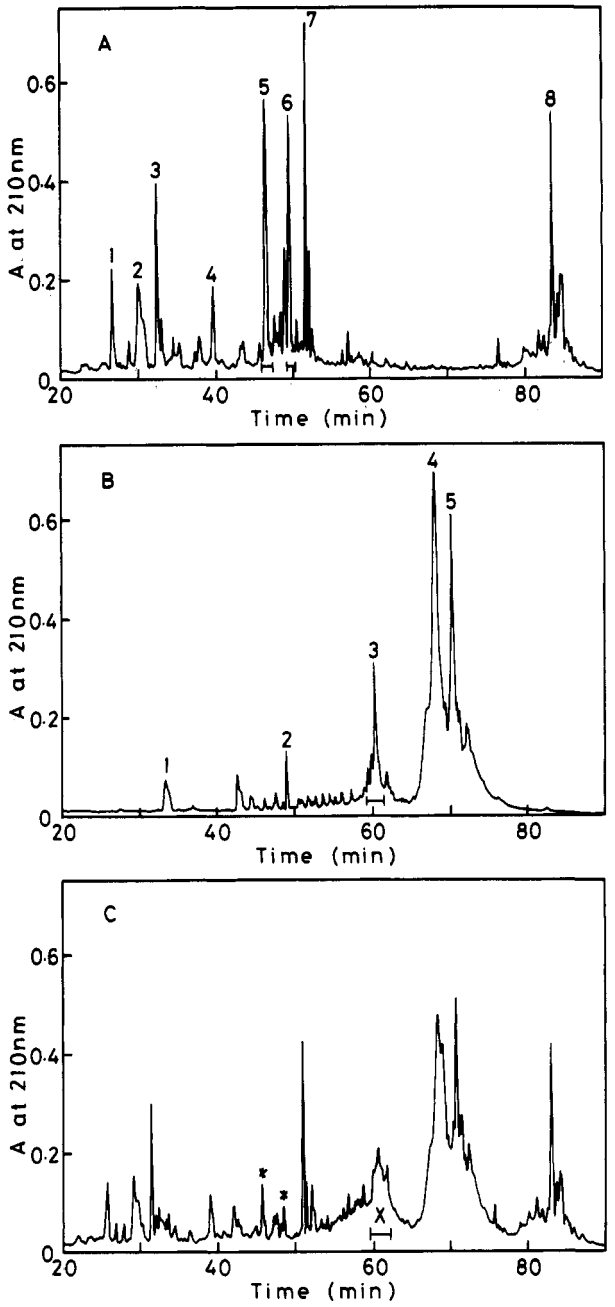

FIGURE 5: Reverse-phase HPLC elution profiles of CNBr digests of the 16K light chain fragment (A), the 24K heavy chain fragment (B), and the 38K cross-linked complex (C). Digests were applied on a reverse-phase HPLC column (0.6 × 25 cm) of Wakosil C_8 and eluted with a linear gradient of acetonitrile concentration with two segments of different slopes: from 0 to 10% solvent B between 0 and 10 min and from 10 to 50% solvent B between 10 and 90 min. The identity of the numbered peaks in panel A are 1, CB10; 2, CB1′, CB1″, CB5, and CB8; 3, CB3; 4, CB7; 5 and 6, CB11; 7, CB2; and 8, CB9. Those in panel B are 1, CN4; 2, CN2; 3, CN6; 4, CN3 and CN2+3; and 5, CN5. See Table I for the exact location of each CB and CN fragment in the regulatory light chain and the 24K heavy chain fragment sequences, respectively. A broad peak designated as X in panel C was pooled and used as the cross-linked fraction. Two peaks significantly reduced by EDC cross-linking are indicated by asterisks.

are probably involved in the cross-linking.

Sequence analysis of peak X1 in Figure 6D revealed the presence of three peptides: residues 151–160 and 164–171 of the regulatory light chain and residues 842–854 of the heavy chain. Of these peptides, only the uncross-linked regulatory light chain peptide consisting of residues 151–160 normally eluted at this position. Further separation of these peptides was performed by diphenyl HPLC. The elution profile shows

Table IV: Sequence Analysis of the Cross-Linked Peptide in Diphenyl HPLC Peak II

	amino acid assignment			
cycle	RLC[a] sequence 164–171	amount (pmol)	HC[b] sequence 842–854	amount (pmol)
1	H	85	L	404
2	G	319	F	351
3	A	298	T	244
4	K	297[c]	K	
5	D	133	V	278
6	K	493[c]	K	
7	D	135	P	206
8	D	145	L	232
9			L	296
10			Q	149
11			V	151
12			T	99
13			R	18

[a] The regulatory light chain. [b] The heavy chain. [c] Lysines were common in both fragments at cycles 4 and 6.

two peaks at 20.4 and 24.1 min (Figure 7, peaks I and II, respectively). Sequence analyses indicated that peak I contains residues 151–160 of the regulatory light chain, whereas peak II contains residues 164–171 of the regulatory light chain and residues 842–854 of the heavy chain. The uncross-linked heavy chain peptide consisting of residues 842–854 eluted at 25.5 min, which was slightly but consistently later than peak II. All of these results indicate that the two peptides in peak II, residues 842–854 of the heavy chain and residues 164–171 of the regulatory light chain, are cross-linked.

As shown in Figure 8, the sequence 842–854 of the heavy chain contains two lysines (Lys-845 and Lys-847) but no glutamic or aspartic acids. Since EDC cross-links between lysine and glutamic or aspartic acid, these two lysines are candidates for cross-linking residue(s). Lysines were located at positions 4 and 6 in both sequences 164–171 of the regulatory light chain and 842–854 of the heavy chain. A sequence analysis revealed that the yield of PTH-lysine at the fourth cycle was only half that at the sixth cycle (Table IV). These results indicate that Lys-845, but not Lys-847, is the cross-linking site to residues 164–171 of the regulatory light chain. On the other hand, the sequence 164–171 of the regulatory light chain includes three aspartic acids (Asp-168, Asp-170, and Asp-171), but yields of PTH-aspartic acids at the fifth, sixth, and eighth cycles were roughly equal (Table IV). It seems unlikely, therefore, that either one of these three residues exclusively participate in the EDC cross-linking to the heavy chain. The simplest explanation for this result is that the side chains of these three aspartic acid residues are involved equally in the cross-linking reaction (Figure 8). Since Asp-171 is the COOH-terminal residue of the regulatory light chain, this residue has two free carboxyl groups. An alternative possible explanation is that the α-carboxyl group of Asp-171 is the only cross-linking site (Figure 8). If it is so, Edman degradation produces a PTH-aspartic acid at the eighth cycle as shown in Table IV.

Two other peaks near the peak X1 in Figure 6D also contained cross-linked peptides. Sequence analysis of peak X2 yielded two simultaneous sequences: residues 168–171 (DKDD) of the regulatory light chain and residues 842–854 (see Table IV) of the heavy chain. The yield of PTH-lysine at the fourth cycle (23 pmol) was much lower than that at the sixth cycle (111 pmol), and yields of PTH-aspartic acids at the first, third, and fourth cycles were roughly equal. These similar results for the cross-linked peptides within peaks X1 and X2 suggest that the same lysine and aspartic acid residues are involved in cross-linking peptides in these two peaks. The

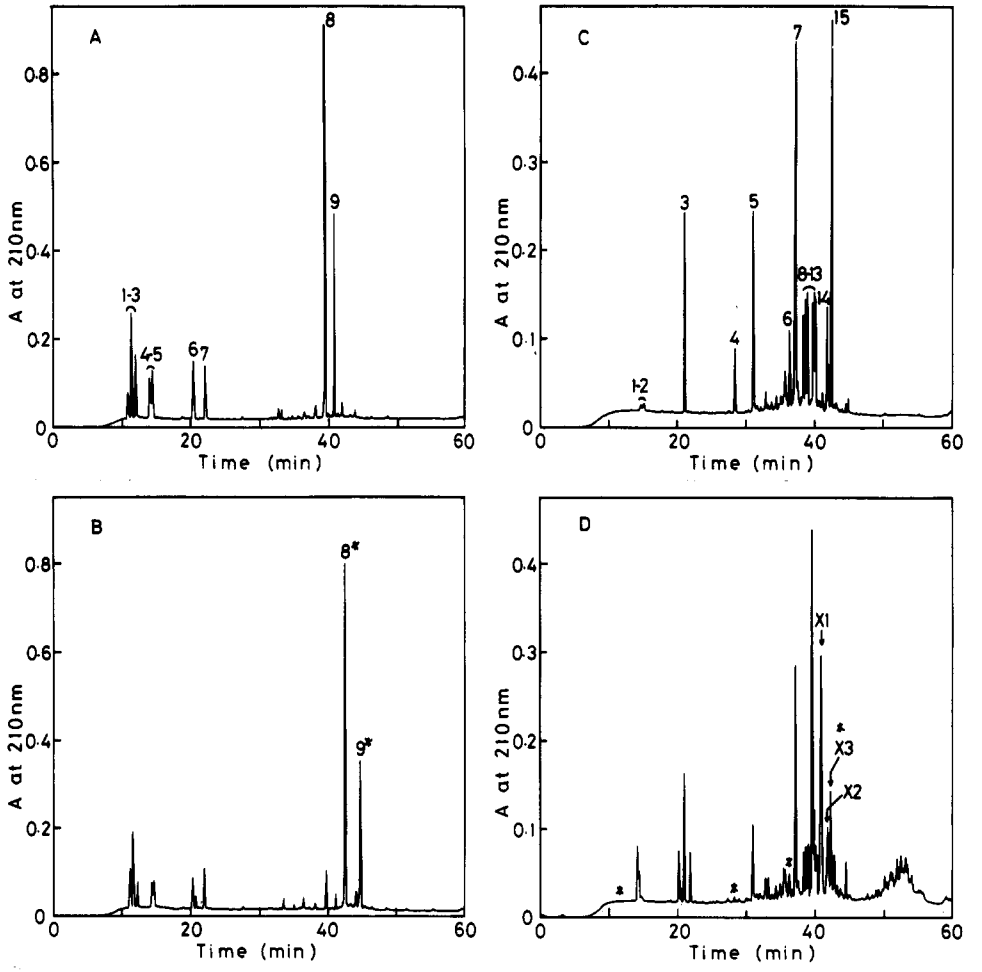

FIGURE 6: HPLC elution profiles of arginylendopeptidase digests of peaks 5 and 6 in Figure 5A (A and B, respectively), peak 3 in Figure 5B (C), and peak X in Figure 5C (D). Samples were applied on a reverse-phase HPLC column (0.46 × 25 cm) of Cosmosil C_{18} and eluted with a linear gradient of acetonitrile concentration from 0 to 45% solvent B between time 0 and 60 min. Numbered peaks in panels A and B represent different arginylendopeptidase peptides obtained from the RLC–CB11 fragment: 1–3, regulatory light chain residues 164–171; 4–5, 142–143; 6, 144–150; 7, 161–163; 8 and 8*, 144–160; and 9 and 9*, 151–160. Those in panel C are arginylendopeptidase peptides obtained from the 24K–CN6 fragment: 1–2, heavy chain residues 834–835; 3, 822–826; 4, 842–845, 5, 827–833; 6, 846–854; 7, 827–835 plus 836–841; 8–13, 836–841; 14, 842–851; and 15, 842–854. Peaks designated as X1, X2, and X3 contained cross-linked peptides. Peaks indicated by asterisks were significantly reduced after EDC cross-linking.

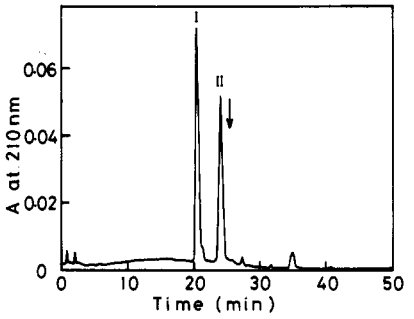

FIGURE 7: Rechromatography of peak X1 with diphenyl HPLC. The separation was carried out on a Vydac 219TP54 column (0.46 × 25 cm). Peptides were eluted with a linear gradient of acetonitrile concentration from 10 to 35% solvent B between 0 and 50 min. The elution time of the uncross-linked fragment consisting of residues 842–854 was indicated by an arrow in this figure. Peak I contains the uncross-linked regulatory light chain fragment consisting of residues 151–160, whereas peak II contains the cross-linked complex between residues 164–171 of the regulatory light chain and residues 842–854 of the heavy chain.

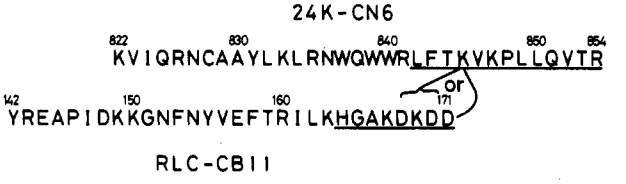

FIGURE 8: Amino acid sequences of the heavy chain and the regulatory light chain CNBr fragments that are cross-linked by EDC. 24K–CN6 and RLC–CB11 sequences correspond to residues 822–854 of the heavy chain (Onishi et al., 1986; Yanagisawa et al., 1987) and residues 142–171 of the regulatory light chain (Maita et al., 1981), respectively. The cross-linked peptide that was isolated after arginylendopeptidase digestion (Figure 7) is underlined.

elution time of peak X3 was identical to that of the uncross-linked regulatory light chain peptide consisting of residues 144–160, but our sequence analysis of this peak revealed the presence of, in addition to residues 144–160, two other fragments: residues 164–171 of the regulatory light chain and residues 842–854 of the heavy chain. This result suggests that peak X3 also contains the same cross-linked complex as peak X1, although the reason why the same cross-linked complex was split into two peaks is not known.

The question of whether Tyr-155 in arginylendopeptidase-digested regulatory light chain peaks 8* and 9* is modified chemically or substituted by isoleucine was investigated by mass spectrometry. The fast atom bombardment mass spectrum of the peak 9* showed an ion signal at m/z = 1324.3 (Figure 9). The observed mass value and the isotopic ion distribution pattern were nearly identical to the theoretical value (1324.5) and the ion distribution of bromotyrosine-

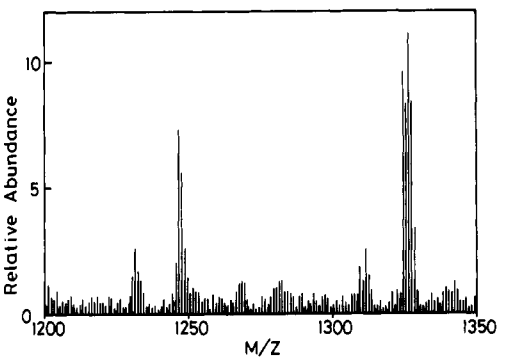

FIGURE 9: Fast atom bombardment mass spectrum of the peak 9* in Figure 6B. The fraction gave an ion signal with mass units = 1324.3, and the isotopic ion distribution within this signal was characteristic for a compound containing one bromine atom. The ion signal with 1246.4 mass units was the result of losing the bromine atom.

containing sequence 151–160 of the regulatory light chain, of which the elemental composition is $C_{57}H_{79}O_{17}N_{15}Br_1$. Another ion signal was observed at m/z = 1246.4 in the spectrum (Figure 9). The isotopic ion distribution indicated the absence of a bromine atom, and the mass value was identical to the theoretical value calculated from the sequence 151–160 of the regulatory light chain containing tyrosine instead of bromotyrosine. This signal is presumably caused by the loss of one bromine atom from the parent ion. All of these results suggest that peak 9* contains the residues 151–160 with brominated Tyr-155. Further evidence that the difference between peaks 8 and 9 and peaks 8* and 9* can be explained by the presence or absence of brominated Tyr-155 comes from comparing the reverse-phase HPLC elution properties of tyrosine and bromotyrosine. The amino acid composition data shown in Table II reveal that the absence of tyrosine was observed simultaneously with the appearance of an additional isoleucine residue. Using synthesized bromotyrosine, we observed that bromotyrosine actually eluted at the position of isoleucine under the conditions employed for our chromatography.

Discussion

The present study demonstrated that a cross-link between S-1 heavy and regulatory light chains was produced by incubating smooth muscle myosin S-1 with EDC. This EDC cross-linking reaction was not affected by the presence of either ATP or F-actin (Figures 1 and 2). As already described in our previous paper (Onishi et al., 1990), EDC is a zero-length cross-linker, and so the two cross-linked amino acids must be almost in contact with each other in the native S-1 molecule. Therefore, it is quite possible that the cross-linked amino acid residues represent one of the association sites between the heavy and the regulatory light chains. Due to its unique cross-linking mechanism, EDC cannot reveal all of the association sites between the heavy and the regulatory light chains. Nevertheless, the present cross-linking data permit us to propose a novel idea for the light-chain binding in gizzard myosin.

The EDC cross-linking site on the heavy chain was determined to be Lys-845. The major regulatory light chain binding site in the myosin heavy chain was reported to be somewhere within the COOH-terminal 24K region of scallop myosin (Szentkiralyi, 1984; Bennet et al., 1984) and vertebrate smooth and skeletal muscle myosins (Sellers & Harvey, 1984; Mitchell et al., 1986). Recently, by using a series of deletions produced by site-directed mutagenesis of heavy chain clones of nematode myosin, Mitchell et al. (1989) mapped the binding site for the regulatory light chain within the COOH terminus of nematode myosin S-1. Deletion of 38 amino acid residues from the COOH terminus of S-1 greatly reduced the regulatory light chain binding to the myosin heavy chain. This deleted region corresponds to residues 805–842 in gizzard myosin. Although different myosins are involved, both our present data and theirs indicate that the regulatory light chain is located near the COOH-terminal end of the S-1 heavy chain.

The EDC cross-linking residue (Lys-845) was only 7 or 10 residues away from the COOH-terminal end of gizzard S-1. A highly homologous sequence K–X–(K/R)–P (residues 845–848 in gizzard myosin) was observed in the neck region of myosins from widely different species (Warrick & Spudich, 1987). From the study of a rod sequence of nematode myosin, McLacklan and Karn (1982) localized the proline in this homologous sequence as the NH_2-terminal boundary of the rod which showed features typical of α-helical coiled-coil structure. Since our cross-linking site was only three amino acid residues away from this proline, the position of the regulatory light chain binding must be at or very close to the S-1–rod hinge portion of the molecule. This conclusion is supported by the results of a previous cross-linking study (Hardwicke et al., 1983) and electron microscopic observations (Vibert & Craig, 1982; Flicker et al., 1983) which have indicated that regulatory light chains of scallop or vertebrate skeletal muscle myosins overlap the neck of the molecule.

Our present results showed a binding property inconsistent with the popular view on the structure of the bound regulatory light chain. A monoclonal antibody directed against the NH_2-terminal region (residues 17–51) of the regulatory (DTNB) light chain of skeletal muscle myosin was shown to bind to the head–rod junction of the molecule (Winkelmann et al., 1983). Photo-cross-linking of two regulatory light chains of scallop myosin at the cysteine near the residue 50 suggests that the NH_2-terminal regions of regulatory light chains are very close to the head–rod junction connecting two myosin heads (Vibert et al., 1985). A proteolytic digestion study of the isolated scallop regulatory light chain suggested the involvement of the COOH-terminal 14 residues of the regulatory light chain in heavy chain binding (Kendrick-Jones & Jakes, 1976). On the basis of these observations and also of the assumption that the bound regulatory light chains are as elongated (10 nm or more long) as the isolated ones (Stafford & Szent-Györgyi, 1978), earlier papers predicted that the heavy and the regulatory light chains were oriented antiparallel in the neck region of myosin heads, so that the COOH-terminal region of the regulatory light chain is located farther away from the head–rod junction of the myosin head (Waller & Lowey, 1985; Vibert et al., 1985; Mitchell et al., 1986). However, our present results indicate that the COOH-terminal end of the regulatory light chains is in contact with the head–rod junction of myosin. Since their conclusion is based on the assumption that the regulatory light chain takes an extended form in the myosin molecule, our data suggest that this assmuption may not be correct.

ATP binding and phosphorylation of the regulatory light chain induced a conformational change in the neck region of gizzard myosin and heavy meromyosin as indicated by electron microscopic studies (Onishi & Wakabayashi, 1982; Trybus et al., 1982; Craig et al., 1983; Onishi et al., 1983; Suzuki et al., 1985) and by digestion studies (Onishi & Watanabe, 1984; Ikebe & Hartshorne, 1984; Suzuki et al., 1988). Muffling of the neck with the tail, reorientation of the heads toward the tail, and the change in papain digestibility of the head–tail junction apparently suggest a conformational change in the hinge region. Since the site of phosphorylation by myosin light chain kinase is Ser-19, very close to the NH_2-terminal end of

the regulatory light chain (Pearson et al., 1984), it is believed that the NH_2-terminal region of the regulatory light chain is important for the regulation of the myosin conformation. However, the present study indicates that the COOH-terminal end of the regulatory light chain directly interacts with the S-1–rod hinge. Although the COOH-terminal end is very far from the phosphorylation site on the linear sequence, we propose that these two sites on the regulatory light chain are very close to each other in the native molecule, and both together are involved in the phosphorylation-induced conformational change in the myosin neck.

The structure of the calmodulin–myosin light chain kinase complex may be relevant to the question of how both the NH_2- and COOH-terminal regions of the regulatory light chain could simultaneously interact with the S-1–rod hinge region. On the basis of the sequence homology, Kretsinger (1980) has suggested that myosin light chains have a structure isologous to calmodulin. Gln-3 and Thr-146 of calmodulin are approximately 37 nm apart in the crystal structure (Babu et al., 1985; Herzberg & James, 1985). Using a site-directed mutant calmodulin in which Gln-3 and Thr-146 have been replaced by cysteins, Persechini and Kretsinger (1988) suggested that in the native calmodulin–enzyme complex, the central α-helix of calmodulin is bent, so that the distance between the two cysteines is much shorter than that seen in the crystal structure. Similarly, the bound regulatory light chain may be bent at the central region. Further studies will be needed to establish the structure of the bound regulatory light chain.

It should be noted that there is some evidence that the invariant proline, together with the sequence (about 53 residues) often attributed to subfragment 2, may actually still be located with the head domain of myosin (Rimm et al., 1989). If this model is correct, the COOH-terminal region of the regulatory light chain could be located farther away from the head–tail junction, and this would alter aspects of the present discussion. In this regard, it is critically important to determine the location of the invariant proline within the myosin molecule.

In the present study, not all possible cross-linked fractions were analyzed. For example, in Figure 6D, a broad, heterogeneous, late-eluting peak appears to be a good candidate for cross-linked peptides, but it was not examined. It is possible that more than one lysine may be cross-linked to activated carboxyl groups. Furthermore, the intramolecular cross-linking within the heavy and/or light chain is also possible. Sequence analyses of HPLC elution peaks in Figure 5A,B indicated that no major peaks contained cross-linked complexes between two CNBr fragments. This cannot exclude, however, the possibility of the cross-linking within the CNBr fragments.

Bromination of tyrosine is probably an artifact of the CNBr treatment, because no bromine atoms could be detected in native gizzard myosin preparations. Residues 144–160 or 151–160 of the regulatory light chain were brominated, whereas residues 142–143 were not (Table II). This result suggests that Tyr-155 is more reactive than Tyr-142 even in a denaturated form of S-1. However, it is unknown why this tyrosine is highly reactive with CNBr.

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Inactivation, Subunit Dissociation, Aggregation, and Unfolding of Myosin Subfragment 1 during Guanidine Denaturation†

Muriel Nozais, Jean-Jacques Bêchet,* and Maurice Houadjeto‡

Laboratoire de Biologie Physicochimique, Unité Associée au CNRS 1131, Bâtiment 433, Université de Paris-Sud, 91405 Orsay, France

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ABSTRACT: The effect of guanidine hydrochloride on ATPase activity, gel filtration, turbidity, exposure of thiol groups, far-UV circular dichroism, and the fluorescence emission intensity of myosin subfragment 1 (S-1) was studied under equilibrium conditions. It was found that the denaturation process involves several intermediate states. The enzymatic activity of S-1 is at first lost at very low concentrations of GdnHCl (lower than 0.5 M). At a slightly higher GdnHCl concentration (about 0.5 M), the light chains dissociate and this dissociation is closely followed by the formation of aggregates between the naked heavy chains of S-1 molecules in the guanidine hydrochloride range of concentrations 0.5–1 M. At GdnHCl concentrations above 1 M, aggregates gradually disappear and S-1 loses its secondary and tertiary structures. These phenomena are partly reversible, and ATPase activity is only partially recovered under highly limited conditions. These results are discussed in relation to the nature of myosin subunit assembly. The head fragment of 20 kDa is thus suggested to be implicated in the binding of light chain to heavy chain and in the self-association of free heavy chains.

Muscle contraction originates from the sliding of myosin filaments on actin filaments, the energy for which is supplied by the hydrolysis of ATP by myosin. Conformational changes in the myosin head have been suggested to occur during this hydrolysis and to be mechanically transmitted to the distant actin-binding site (Botts et al., 1989; Mornet et al., 1989), allowing the conversion of chemical energy into the mechanical energy of contraction.

Experiments of protein denaturation–renaturation may help us to understand how a multidomain oligomeric protein, such as the myosin head, folds and how its subunits and domains acquire stability [for a review, see Jaenicke (1987)]. The portion of myosin studied in this work is the myosin head or myosin subfragment 1 (S-1)[1] obtained after chymotryptic proteolysis. It consists of one essential light chain of either 21 or 16.5 kDa and one heavy chain (approximately 95 kDa). The latter is usually considered to comprise three domains, i.e., the consecutive 25-, 50-, and 20-kDa fragments. The effect of guanidine hydrochloride on ATPase activity, gel filtration, turbidity, exposure of thiol groups, far-UV circular dichroism, and the fluorescence emission intensity of myosin subfragment 1 was studied, and the transition curves of these different signals were established as a function of the concentration of the denaturant for the processes of denaturation and renaturation.

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* Corresponding author.
‡ Present address: INSERM U128, CNRS, BP 5051, 34033 Montpellier Cedex, France.

[1] Abbreviations: S-1, myosin subfragment 1; GdnHCl, guanidine hydrochloride; HC, heavy chain; LC, light chain; A1, higher molecular mass alkali (or essential) light chain; A2, lower molecular mass alkali (or essential) light chain; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); TNB, 5-thio-2-nitrobenzoic acid.

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