Effect of Nucleotides and Actin on the Intramolecular Cross-Linking of Myosin Subfragment-1†

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ABSTRACT: The heavy chain of myosin subfragment-1 (S1) is cleaved by limited trypsinolysis into three fragments, 27, 50, and 20 kDa—aligned in this order from the N-terminus. The tertiary structure of the molecule is essentially not affected by trypsinolysis. The spatial relations between the various regions of the molecule and the nucleotide- and actin-induced intramolecular movements were studied by cross-linking tryptic S1 with *N*-(ethoxycarbonyl)-2-ethoxy-1,2-dihydroquinoline (EEDQ), 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (EDC), phenylenediglyoxal (PDG), and glutaraldehyde. The formation of cross-linked products was monitored by SDS–PAGE, using the fluorescent probes 9-anthronitrile and *N*-(iodoacetyl)-*N'*-(5-sulfo-1-naphthyl)ethylenediamine (IAEDANS), which specifically label the 27- and 20-kDa fragments, respectively. The reaction with the cross-linkers leads to the formation of 50-kDa/20-kDa, 27-kDa/20-kDa, 27-kDa/50-kDa, and 20-kDa/light chain cross-linked products. Of these, the most intensive was the formation of the 50-kDa/20-kDa products, which appeared as a doublet on the SDS–PAGE with all the cross-linkers. This indicates that the interface between the two fragments is rather extended. The presence of MgATP or MgADP promoted the formation of the 20-kDa/50-kDa cross-linked products, especially with the lower electrophoretic mobility band, when EEDQ was used as a cross-linker. With PDG as a cross-linker, MgATP also affected the cross-link formation between the 20-kDa fragment and the light chains whereas it had no influence on the formation of other products. On the other hand, the effect of actin on the cross-linking with the various cross-linkers was quite extensive, and it was manifested in the reduction of cross-link formation between the various S1 domains. It is concluded that both nucleotides and actin induce intramolecular movements in S1 and that the nucleotide-induced movements are more restricted than those induced by actin, which extend to larger regions of the molecule.

Myosin and actin are two cardinal proteins of muscle whose interaction with each other coupled with the hydrolysis of ATP constitute the molecular basis of contraction. The head segment of myosin, called S1,[1] contains the distinct actin- and nucleotide-binding sites of myosin (Mueller & Perry, 1962) and is sufficient together with ATP and actin to generate movement *in vitro* (Toyoshima et al., 1987). It is, therefore, obvious that the structural and functional characterization of S1 has a great significance.

In addition to the very recent progress in S1 crystallography (Rayment et al., 1993a), studies using both limited proteolysis and cross-linking have contributed significantly to the description of the S1 structure. It has been shown that the heavy chain of S1 is cleaved by limited tryptic digestion into three major fragments, namely, 27, 50, and 20 kDa [aligned from the N-terminus in this order (Balint et al., 1978)]. Following trypsinolysis, the fragments remain connected to each other by secondary forces, which keep the tertiary structure of the molecule essentially intact. The proteolytic fragments of S1 heavy chain were assumed to be domains connected to each other by short, loose, protease-sensitive connectors (Mornet et al., 1981). The fragments have become a valuable framework to which the nucleotide- and actin-binding sites and other specific functionalities can be assigned. It has been shown that the "SH_1" and "SH_2" reactive thiols are located on the 20-kDa fragment (Balint et al., 1978) and that the reactive lysyl residue is located on the 27-kDa fragment (Mornet et al., 1980). The nucleotide-binding site of S1 has been assigned to the C-terminal region of the 27-kDa fragment and to the N-terminal region of the 50-kDa fragment by trapping analogues of ATP (Szilagyi et al., 1979; Mahmood & Yount, 1984) and phosphate (Cremo et al., 1989; Muhlrad et al., 1991) at the active site. A distinct actin-binding site has been located at the 50-kDa/20-kDa junction and on the 50-kDa fragment by various methods (Mornet et al., 1979a; Chaussepied & Morales, 1988) including cross-linking (Sutoh, 1983).

It is generally assumed that the contractile force originates from the hydrolysis of ATP, which takes place at the nucleotide-binding site of S1. The energy released during the ATP hydrolysis is transferred through distortions in the tertiary structure of S1 to the distinct actin-binding site, where tension is generated at the actin–S1 interface. Thus, S1 serves basically as an energy transducer in the process (Morales & Botts, 1979). The intermediates of the ATP cycle induce in S1 specific conformational changes which define the actin–S1 relationship at each step. Actin binding also produces structural changes which affect ATP binding and hydrolysis at the nucleotide-binding site. Thus, there is a two-way communication between the two binding sites. The nucleotide- and actin-induced conformational changes are manifested as

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[1] Abbreviations: S1, myosin subfragment-1; DTE, dithiothreitol; PMSF, phenylmethanesulfonyl fluoride; EEDQ, *N*-(ethoxycarbonyl)-2-ethoxy-1,2-dihydroquinoline; EDC, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide; IAEDANS, *N*-(iodoacetyl)-*N'*-(5-sulfo-1-naphthyl)-ethylenediamine; ANN, 9-anthronitrile; PDG, *p*-phenylenediglyoxal; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

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altered reactivity of functional groups, altered spectral characteristics of internal and external reporter groups, and altered proteolytic susceptibility [for a review, see Audemard et al. (1988)]. These studies indicate that conformational changes take place in all three domains of the S1 heavy chain. However, they do not describe the extent and the direction of the nucleotide-induced internal movements in S1. Efforts were made to characterize quantitatively these movements by measuring distances between S1 functionalities in the presence and in the absence of nucleotides with fluorescence resonance energy transfer and cross-linking. The cross-linkers employed in these studies specifically reacted with either the SH_1 or the SH_2 reactive thiols—both located on the 20-kDa fragment of S1—making these residues one of the "anchor" points in the cross-link. It was shown by these studies that the distance between SH_1 (Cys-707) and SH_2 (Cys-697) [numbering is according to Tong and Elzinga (1983)] changes under the influence of nucleotide binding (Burke & Reisler, 1977; Dalbey et al., 1983). In the absence of nucleotide, the SH_1 thiol was cross-linked to Glu-88, which is near the N-terminus, while in the presence of nucleotide it also cross-linked to a region located 57–60 kDa away from the N-terminus of S1 (Lu & Wong, 1989). On the other hand, the SH_2 thiol cross-links by 4′-maleimidobenzophenone to Arg 239 at the N-terminal region of the central domain in the absence of MgADP and, in its presence, the binding shifts to the direction of the C-terminus (Rajasekharan et al., 1989; Agarwal et al., 1991). Using another cross-linker, *p*-nitrophenyl iodoacetate, SH_2 was cross-linked to Lys 184 or Lys 189 in the absence of MgADP, but not in its presence (Sutoh & Hiratsuka, 1988). MgATP significantly decreases the distance between SH_2 and the 50-kDa/20-kDa connector region as indicated by fluorescence energy-transfer studies (Kasprzak et al., 1989). On the basis of these and other results, an attempt was made to model the intramolecular movements (Botts et al., 1989). Accordingly, at least two movements occur upon the addition of nucleotide: (a) SH_2 moves toward SH_1 and away from the nucleotide-binding site and (b) a strand containing these two thiols in the C-terminal domain and a parallel strand in the central domain move toward each other. Even less is known about the actin-induced intramolecular S1 movements. There are results which show that the cross-link formation by benzophenone iodoacetamide between SH_1 or SH_2 and some region on the 50-kDa fragment is inhibited in the presence of actin (Lu et al., 1987), thus implying that the distance between these two regions increased as a consequence of actin binding. The available data are not sufficient to characterize quantitatively the nucleotide- and actin-induced intramolecular movements in S1, and further studies are needed to solve this important problem of contractility.

In this paper, the nucleotide- and actin-induced intramolecular movements are studied by cross-linking the S1 fragments obtained following limited trypsinolysis. The four cross-linkers used in this work—EDC, EEDQ, PDG, and glutaraldehyde—do not preferentially target thiol groups, and therefore, the present study, unlike most of the former ones, is not confined to cross-links in which the SH_1 or the SH_2 thiol is one of the "anchor" points. The S1 fragments participating in cross-link formation were detected by specifically labeling the 20- and 27-kDa fragments with IAEDANS and ANN, respectively. The results showed cross-linking between the three tryptic fragments of S1. The major products of the reaction were the 50-kDa/20-kDa cross-link products, which appeared as a doublet on SDS–PAGE in most cases. With EEDQ as a cross-linker, MgATP or MgADP promoted the formation of the 50-kDa/20-kDa products, which indicates that nucleotide-induced intramolecular movements are taking place in the S1 structure. Actin also induced intramolecular movements, which are manifested in decreased cross-link formation between the various domains of S1, and these movements were propagated to larger regions of the molecule than those induced by nucleotides.

MATERIALS AND METHODS

Chemicals. ATP, ADP, chymotrypsin, trypsin, soybean trypsin inhibitor, DTE, PMSF, EDC, glutaraldehyde, and IAEDANS were from Sigma Chemical Co. EEDQ was from Aldrich Chemical Co. PDG and ANN were purchased from Molecular Probes Inc. All other chemicals were of analytical grade.

Preparation of Proteins. Myosin and actin were prepared from back and leg muscles of rabbit by the methods of Tonomura et al. (1966) and Spudich and Watt (1971), respectively. S1 was obtained by the digestion of myosin filaments with chymotrypsin at a 300:1 (by mass) ratio (Weeds & Taylor, 1975). The digestion was stopped using 0.1 mM PMSF. In some cases the S1 isoforms, S1(A1) and S1(A2), were separated on a WCX ZORBAK cation-exchange column in an LKB HPLC system. The separation was performed by a 0–40% gradient at 4 °C. Solution A, 30 mM Tris-HCl, pH 7.7; solution B, 500 mM NaCl and 30 mM Tris-HCl, pH 7.7; S1(A1) and S1(A2) eluted at 15% and 24% of solution B, respectively. Protein concentrations were obtained by absorbance, using an A (1%) at 280 nm of 7.5 for S1 and an A (1%) at 290 nm of 6.3 for actin. Molecular masses were assumed to be 115 and 42 kDa for S1 and actin, respectively.

Labeling of S1. S1 was labeled with the fluorescence probe IAEDANS, according to Takashi (1979). This reagent specifically reacts with the SH_1 thiol (Cys-707) on the 20-kDa fragment of the S1 heavy chain. Briefly, a 3.5 mM IAEDANS stock solution was freshly prepared in 50 mM TES, pH 7.0. S1 was incubated with a 10-fold molar excess of IAEDANS in the above buffer at 0 °C in the dark for 40 min. The reaction was quenched by the addition of 7 mM DTE, and the unreacted IAEDANS was removed by dialyzing the protein solution against the buffer used for cross-linking at 4 °C for 24 h. S1 was specifically labeled on the 27-kDa N-terminal fragment with the fluorescence probe ANN according to Hiratsuka (1989). ANN was dissolved at a concentration of 40 mM in dimethylformamide. S1 was incubated in a 20 mM MES buffer, pH 7.0, for 90 min at 25 °C in the dark, with a 20-fold molar excess of ANN. A total of 2 mM MgATP was added to the mixture before the incubation was started. The reaction was stopped by the addition of 17 mM Tris–malelate buffer, pH 7.0. The excess of reagent was removed by dialysis against the buffer used for cross-linking at 4 °C for 24 h. Following dialysis, S1 was centrifuged at 15000*g* for 15 min in order to remove precipitated material.

Tryptic Digestion of S1. S1 was digested with trypsin in the buffer used for cross-linking with the various cross-linkers at a 1:50 (by mass) ratio to S1. Trypsinolysis was performed at 25 °C for 30 min. Digestions were terminated by adding soybean trypsin inhibitor at a 2:1 (by mass) ratio to trypsin.

Cross-Linking. All cross-linking reactions were performed in the presence of either 4 mM $MgCl_2$, 4 mM MgATP, or 4 mM $MgCl_2$ plus 60 μM actin. The details of the reaction conditions for each cross-linkers are given in the following.

(*A*) *EDC.* A total of 30 μM tryptic S1 or S1 was incubated with 10 mM EDC (freshly dissolved in H_2O), in 50 mM KCl, and 10 mM TES buffer, pH 7.0, for 30 min at 25 °C. The reaction was terminated by adding 50 mM 2-mercaptoethanol

for assaying ATPase or 0.25 vol protein sample buffer [9.4% SDS, 3.1% 2-mercaptoethanol, 31 mM Tris-HCl buffer (pH 6.8), 31% glycerol, 0.005 bromophenol blue] for analysis by SDS–PAGE.

(*B*) *EEDQ*. A total of 30 μM tryptic S1 or S1 was incubated with 1 mM EEDQ (freshly dissolved in absolute ethanol) for 30 min at 25 °C in 50 mM KCl and 10 mM TES buffer, pH 7.4. The reaction was terminated by adding 2 mM DTE or 0.25 vol protein sample buffer for ATPase assays and for product analysis by SDS–PAGE, respectively.

(*C*) *PDG, Carried out According to Bonet et al. (1988)*. A total of 30 μM tryptic S1 or S1 was incubated with 5 mM PDG (freshly dissolved in 50% methanol + 50% 0.1 M potassium bicarbonate buffer, pH 8.3) in a 0.1 M potassium bicarbonate buffer, pH 8.3, for 30 min at 25 °C. The cross-linking reaction was terminated by adding 50 mM 2-mercaptoethanol for assaying ATPase activity or 0.25 vol protein sample buffer for product analysis by SDS–PAGE.

(*D*) *Glutaraldehyde, Performed According to Bertrand et al. (1988)*. A total of 30 μM tryptic S1 or S1 was incubated with 1 mM glutaraldehyde in 100 mM Hepes buffer, pH 8.0, at 25 °C for 30 min. The reaction was stopped by adding 50 mM 2-mercaptoethanol for determination of ATPase activity or 0.25 vol protein sample buffer for analysis by SDS–PAGE.

Measurement of Binding of S1 to F-Actin by Sedimentation. Samples of 3 μM S1 and 6 μM F-actin in 1 mM $MgCl_2$, 10 mM TES, pH 7.0, and 50 mM or 100 mM NaCl were incubated at 25 °C for 10 min. The samples were centrifuged at 100 000 rpm for 15 min at 25 °C in a Beckman TL-100 ultracentrifuge. Aliquots were taken from the supernatant, mixed with 0.25 vol protein sample buffer, and analyzed by SDS–PAGE. The optical densities of the protein bands on the Coomassie Brilliant Blue-stained gels were determined with Molecular Dynamics personal densitometer.

ATPase Assays. Actin-, Mg^{2+}-, K^+(EDTA)-, and Ca^{2+}-activated ATPase activities (micromoles of phosphate per milligram of S1 or tryptic S1 per minute) were calculated from the inorganic phosphate produced, measured according to Fiske and Subbarow (1925). The reaction was performed at 25 °C on 1-mL aliquots taken at various time intervals. Incubation times were chosen so that no more than 15% of the ATP was hydrolyzed. For actin-activated ATPase, the assay contained 0.1 μM S1, 0.5 μM F-actin, 2 mM $MgCl_2$, 1 mM ATP, 20 mM imidazole buffer, pH 7.0, and no actin for Mg^{2+}-modulated ATPase. For K^+(EDTA)-activated ATPase, the reaction mixture contained 0.1 μM S1 or tryptic S1, 5 mM EDTA, 600 mM KCl, and 50 mM Tris-HCl buffer, pH 8.0; for Ca^{2+}-activated ATPase, the same conditions were used except that the mixture contained 6 mM $CaCl_2$ instead of EDTA.

SDS–PAGE. Electrophoretic analysis of the samples was carried out on 7–18% polyacrylamide slab gels. First, the fluorescent probe-labeled peptide bands were visualized by near-UV light, and then the gel was stained by Coomassie Brilliant Blue. Molecular masses of the peptide bands were estimated by comparing their electrophoretic mobilities with those of authentic markers.

RESULTS

Intramolecular Cross-Link Formation between S1 Heavy Chain Fragments with EEDQ Cross-Linker. EEDQ is a zero-length cross-linker, which activates carboxyl groups in a hydrophobic environment. The activated carboxylate reacts subsequently with an amino group, which leads to the formation of a covalent cross-link, a peptide bond, between the two reacting moieties. We observed cross-link formation in the presence of EEDQ between the various subunits, including tryptic heavy chain fragments and light chains of S1 (Figure 1). The two S1 isoforms, S1(A1) and S1(A2), used in this study were labeled with the fluorescence probe IAEDANS, which specifically reacts with the SH_1 thiol located on the 20-kDa fragment. The fluorescence labeling greatly facilitates the identification of subunits participating in the various cross-linked products. The formation of the cross-linked products and the effect of nucleotides and actin on the cross-linking are summarized in Table 1. On the Coomassie Brilliant Blue-stained gel (Figure 1A), two cross-linked products with apparent molecular masses of 85- and 75-kDa were easily recognizable. According to the fluorescent picture (Figure 1B), both products contained the 20-kDa fragment that also participates in the formation of the 48-, 46-, and 44-kDa products, which can be detected only in the fluorescent picture. We assume, on the basis of the electrophoretic mobility of the cross-linked products, that the second participant in both the 85- and 75-kDa product is the 50-kDa heavy chain fragment. [Since the sum of the molecular masses of the 50- and 20-kDa fragments is 70 kDa, one may suspect that the 85-kDa band also contains a light chain in addition to the above-mentioned two heavy chain fragments. However, this is unlikely because the electrophoretic mobility of the 85-kDa band was identical upon cross-linking S1(A) or S1-(A2) isoforms.] Since the 46-kDa band appears only with the S1(A1) while the 44-kDa band only appears with the S1(A2) isoform, it is assumed that second participant in the 46- and 44-kDa products are the A1 and A2 (or by other names LC1 and LC3) light chains, respectively. (Since the LC1 light chain is degraded to LC1′ by trypsin, it is the LC1′ that actually participates in the cross-link.) On the other hand, the 48-kDa product appears in the case of both S1 isoforms and on the basis of this finding, together with the electrophoretic mobility of the band, we conclude that the second participant of the 48-kDa cross-linked product is the 27-kDa N-terminal fragment of the S1 heavy chain. This assumption is supported by the results of the cross-linking of the ANN-labeled tryptic S1 (Figure 1C, lanes c–e)—the fluorescent probe ANN specifically labels the 27-kDa fragment (Hiratsuka, 1989)—which unambiguously indicates that the 48-kDa band is a 27kDa/20kDa cross-linked product. The appearance of a faint 78kDa band on the same lane is due too the formation of a 27-kDa/50-kDa cross-linked product. Moreover, this electrophoretogram supports the assumption that the 46- and 44-kDa bands are 20-kDa/light chain cross-linked products, since they appear only on the fluorescent picture of the cross-linked IAEDANS-labeled tryptic S1 but not on that of the ANN-labeled species (Figure 1C). The effect of nucleotides on the cross-link formation was also studied in the experiment presented in Figure 1. It was found that in the presence of MgATP or MgADP the formation of both the 85- and the 75-kDa 50-kDa/20-kDa cross-linked products was enhanced and that the increase in the intensity of the 85-kDa band was especially significant. The appearance of the other cross-linked product bands was not affected by the presence of nucleotides.

The effect of actin on the cross-link formation with EEDQ was also studied (Figure 2). Actin was found to strongly inhibit all EEDQ cross-linking between the S1 segments (Figure 2A,B, lanes d and e). The inhibitory effect of actin is not due to competition of actin with S1 for the cross-linker because EEDQ was added in a large excess, and relatively small amounts of actin–S1 and actin–actin cross-linked products were formed during the reaction. Moreover, in a control experiment EEDQ was incubated with actin at 25 °C

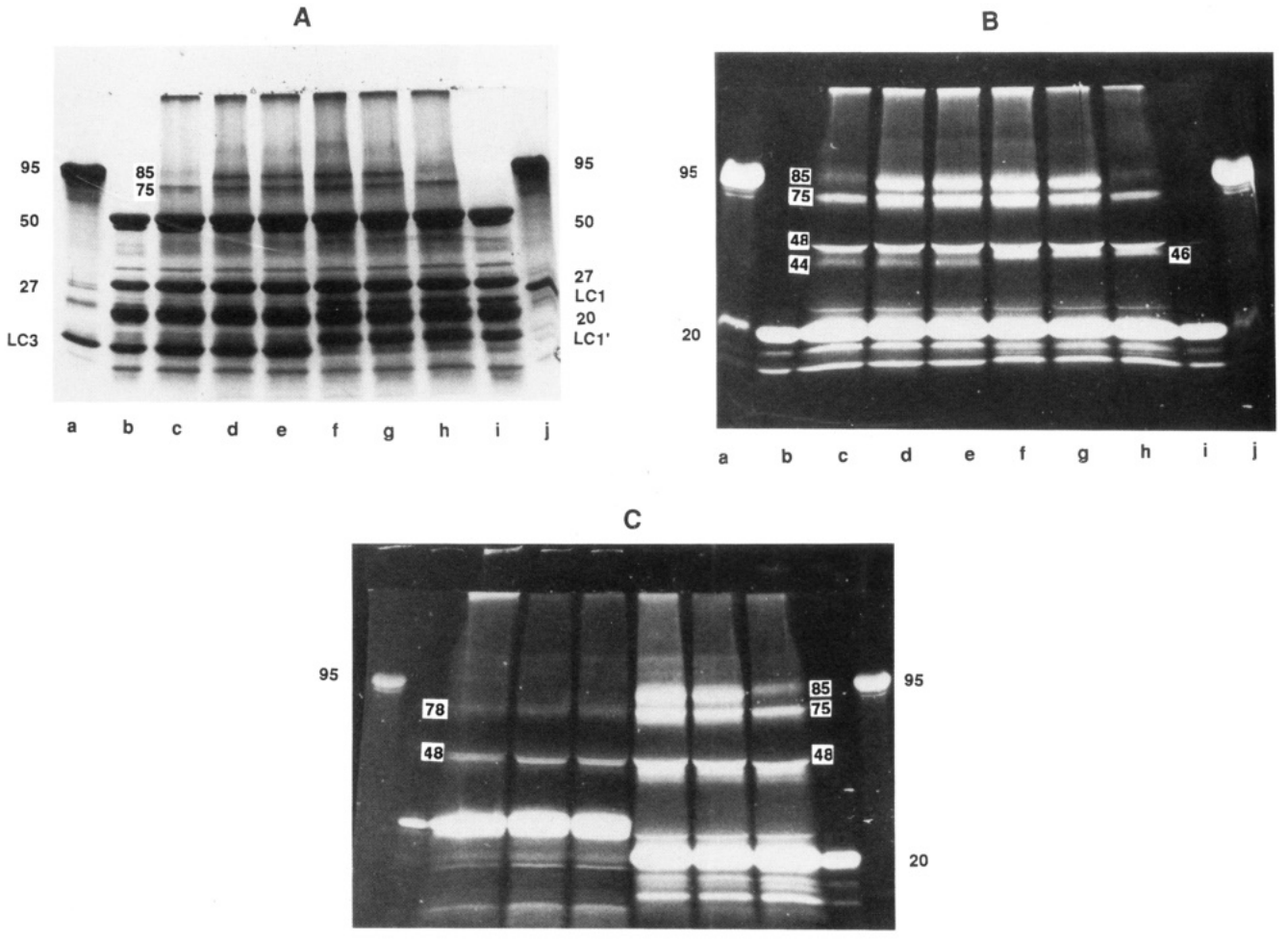

FIGURE 1: Cross-linking of tryptic S1 with EEDQ. S1 was labeled with IAEDANS or ANN, digested with trypsin, cross-linked with EEDQ, and analyzed by SDS–PAGE as described in Materials and Methods. (Panels A and B) Tryptic S1 (A1) or tryptic S1 (A2) labeled with IAEDANS. (A) Coomassie blue stained; (B) fluorescent electrophoretogram. Lanes: a–e, S1(A2); f–j, S1(A1); a and j, undigested S1(A2) and S1(A1); b and i, tryptic S1(A2) and tryptic S1(A1); c–h, cross-linked tryptic S1(A2) and tryptic S1(A1); c and h, cross-linking in the presence of $MgCl_2$; d and g, cross-linking in the presence of MgATP; e and f, cross-linking in the presence of MgADP. (Pancel C) S1 labeled with ANN (lanes a–e) or IAEDANS (lanes f–j). Lanes: a and j, undigested S1; b and i, tryptic S1; c–h, cross-linked tryptic S1; c and h, cross-linking in the presence of $MgCl_2$; d and g, cross-linking in the presence of MgATP; e and f, cross-linking in the presence of MgADP. Vertical numbers, molecular mass in kilodaltons; LC, light chain.

for 10 min prior to addition of tryptic S1, and this preincubation did not affect the extent of cross-link formation. Similar experiments were carried out with the same results also with the EDC glutaraldehyde and PDG cross-linkers, which seems to exclude the possibility of the actin–S1 competition for the cross-linkers.

The cross-linking reaction with EEDQ was found to affect the ATPase activity of S1 and tryptic S1. The K^+(EDTA)-, Ca^{2+}-, Mg^{2+}-, and actin-activated ATPase activity of S1, cross-linked in the presence or in the absence of MgATP, were assayed and compared to the activity of the control, nontreated S1 (Table 2). The K^+(EDTA)- and actin-activated ATPase of S1 considerably decreased as a result of the cross-linking, and the decrease was more pronounced when S1 was cross-linked in the presence of MgATP. The Mg^{2+}-modulated ATPase activity of S1 was essentially not affected by the cross-linking, while the Ca^{2+}-activated S1 ATPase was slightly increased. Essentially the same results were obtained by assaying the K^+(EDTA)- and Ca^{2+}-activated ATPase activities of cross-linked tryptic S1. We investigated also the effect of the EEDQ treatment on the binding of S1 to actin in a sedimentation assay. Essentially no change was observed in the amount of S1 sedimented with actin as a result of the cross-linking (Table 3).

Intramolecular Cross-Link Formation in S1 with EDC as Cross-Linker. The cross-link formation between S1 fragments was also studied using the heterobifunctional zero-length cross-linker, EDC, which activates carboxyl groups in an hydrophilic environment and couples them to amino groups by forming peptide bonds (Figure 3). S1 was labeled with IAEDANS or ANN, digested with trypsin, and reacted with EDC, and the products were analyzed by SDS–PAGE (results are summarized in Table 1). On the Coomassie Brilliant Blue-stained electrophoretogram, a new band appears following the EDC treatment with the apparent molecular mass of 73-kDa (Figure 3A). The analysis of the fluorescent picture (Figure 3B) indicates the formation of the following cross-linked products: 75-kDa band (faint), a 27-kDa/50-kDa product; 73kDa band, a 20-kDa/50-kDa product; 48-kDa band, a 27-kDa/20-kDa product; 45-kDa band, a light chain/20-kDa cross-linked product. In some gels (not shown) a 96-kDa band, a 50-kDa/20-kDa cross-linked product, can also be seen. The 97–96-kDa, the 75–73-kDa, and the 48–45-kDa bands cannot be resolved in the Coomassie Brilliant Blue-stained gel and appear as wide bands or doublets in the picture. The presence of MgATP during the reaction does not influence the formation of the cross-linked products. On the other hand, actin has a dramatic influence on cross-link formation with EDC between the 50- and 20-kDa fragments of S1. The 73-kDa major band almost completely disappeared

Table 1: Intramolecular Cross-Linked Products Formed in the Reaction of S1 with Various Cross-Linkers and the Effect of MgATP and Actin on Cross-Link Formation

cross-linker	composition of cross-link	apparent molecular mass (kDa)	effect of MgATP[a]	effect of actin[a]
EEDQ	50-kDa/20-kDa	85	↑↑↑	↓↓↓
	50-kDa/20-kDa	75	↑	↓↓
	27-kDa/50-kDa	78		↓
	27-kDa/20-kDa	48		↓↓
	20-kDa/LC1′[b]	46		↓↓↓
	20-kDa/LC3	44		↓↓↓
EDC	50-kDa/20-kDa	73		↓↓↓
	27-kDa/50-kDa	75		↓↓
	27-kDa/20-kDa	48		
	20-kDa/LC[c]	45		↓↓↓
glutaraldehyde	50-kDa/20-kDa	96		↓↓↓
	50-kDa/20-kDa	74		↓↓↓
	27-kDa/50-kDa	96		
	27-kDa/50-kDa	74		
	27-kDa/20-kDa	48		
PDG	50-kDa/20-kDa	96	↓	↓
	50-kDa/20-kDa	73		↓
	27-kDa/50-kDa	74		
	20-kDa/LC1′[b]	42		
	20-kDa/LC3	38	↓	

[a] (↑) increased and (↓) decreased cross-link formation; the number of arrows indicates the extent of change in formation. [b] LC1′ is produced from LC1 by tryptic digestion. [c] Probably contains both LC1′ and LC3 light chains.

in the presence of actin in the Coomassie Brillant Blue-stained picture (Figure 3A), while two new major bands appeared with an apparent molecular mass of 68 and 97-kDa. Of the two bands, only the 68-kDa is fluorescent with IAEDANS labeling (Figure 3B, lane e). Combined with their electrophoretic mobilities, this indicates that the 68- and 97-kDa bands correspond to the 20-kDa/actin and the 50-kDa/actin cross-linked products, respectively. The 20-kDa/light chain product, the 45-kDa band on the IAEDANS-fluorescent gel, and the faint 75-kDa band, a 27-kDa/50-kDa product, are also eliminated in the presence of actin, but the formation of the 48-kDa band, a 27-kDa/20-kDa product, does not seem to be affected (Figure 3B, lanes e and f).

Next, we studied the effect of the EDC treatment on the ATPase activity of S1 and tryptic S1. The K^+(EDTA)-activated ATPase activity of S1 is strongly inhibited while its Ca^{2+}-activated ATPase is sharply increased following the EDC reaction. The change in both activities was more pronounced when the reaction took place in the absence of MgATP (Table 2). Essentially the same results were obtained also with tryptic S1 (not shown). The actin-activated ATPase of S1 decreased while its Mg^{2+}-modulated ATPase increased as the consequence of the reaction. The increase in Mg^{2+}-ATPase was much more modest than in the case of Ca^{2+}-ATPase. The binding of S1 to actin, measured in a sedimentation assay, was found to be slightly decreased following the EDC treatment (Table 3).

Intramolecular Cross-Link Formation between S1 Heavy Chain Fragments with Glutaraldehyde Cross-Linker. Glutaraldehyde is a homobifunctional protein cross-linker that specifically reacts with amino groups (Habaeeb & Hiramoto, 1968). This reagent was used earlier to study cross-link formation in the myosin head (Hiratsuka, 1986). In this work, we used the reagent to detect cross-link formation between various S1 fragments and to study the nucleotide- and actin-induced intramolecular movements in S1 (results are summarized in Table 1). First, we monitored the cross-link formation between the S1 heavy chain fragments following glutaraldehyde treatment of IAEDANS- and ANN-labeled tryptic S1 in the presence and absence of MgATP (Figure 4): On the Coomassie Brilliant Blue-stained gel, we observed the appearance of two cross-linked products with apparent molecular mass of 74 and 96 kDa (Figure 4A). According to the fluorescent picture (Figure 4B, lanes c and d; the Coomassie blue-stained and fluorescence gels are unrelated) both bands contain the 20-kDa fragment. The same bands also show faint ANN fluorescence (Figure 4B, lanes g and h), which would indicate that they also contain the 27-kDa fragment. However, the electrophoretic mobility of the bands implies the presence of the 50-kDa fragment in the products. The electrophoretic mobility of the lower band, corresponding to a molecular mass of 74 kDa, seems to exclude the possibility that it contains all the three S1 heavy chain fragments, so it is assumed to be a 50-kDa/20-kDa cross-linked product. The 96-kDa band can accommodate all three fragments on the basis of electrophoretic mobility; however, its very faint ANN fluorescence, which is even weaker than that of the 74-kDa band, implies that it also contains mainly a 50-kDa/20-kDa product. Therefore, we assume that the major components of the 74- and 96-kDa bands are the two distinct 50-kDa/20-kDa cross-linked products whose electrophoretic mobilities overlap with those of 27-kDa/50-kDa products, which are present in small quantities. In addition, a 48-kDa fluorescent band appears after cross-linking in the electrophoretogram of both the IAEDANS- and the ANN-labeled tryptic S1 (Figure 4B), which is due to the formation of a 27-kDa/20-kDa cross-linked product. The intramolecular cross-link formation of S1 with glutaraldehyde proceeds, under the conditions of the experiment presented in Figure 4, similarly in the presence and absence of MgATP. The presence of actin was found to prevent the appearance of the 74- and 96-kDa IAEDANS-labeled fluorescent bands, corresponding to the two distinct 50-kDa/20-kDa cross-linked products (Figure 4B, lane e), but does not affect the formation of the minor 27-kDa/50-kDa and 27-kDa/20-kDa products (Figure 4B, lanes h and e).

The ATPase activity of S1 and tryptic S1 was found to be affected by the reaction with glutaraldehyde (results with S1 presented in Table 2; tryptic S1 results are not shown). Both the Ca^{2+}- and the K^+(EDTA)-activated ATPase activities of S1 and tryptic S1 decreased as the consequence of the reaction, and in most of the cases, the decrease was stronger when the proteins were treated in the absence of MgATP. The actin-activated ATPase of S1 essentially lost while its Mg^{2+}-modulated ATPase activity increased, following the reaction with glutaraldehyde. The capability of S1 to bind to F-actin under rigor conditions—studied by a sedimentation assay (see Materials and Methods)—was severely decreased as a consequence of the cross-linking with glutaraldehyde (Table 3).

Intramolecular Cross-Link Formation in S1 with PDG Cross-Linker. PDG is a homobifunctional cross-linker that specifically reacts with arginine residues (Wagner & Gaussen, 1975). We used this reagent to form intramolecular cross-links between various fragments of tryptic S1 labeled with the fluorescence probes IAEDANS or ANN. In addition, we studied the effect of MgATP and actin on the intramolecular cross-link formation (the results are summarized in Table 1). The products of the cross-linking reaction were analyzed by SDS–PAGE (Figure 5). The electrophoretograms were viewed first by UV light to get the fluorescence picture and then stained by Coomassie Brilliant Blue. On the Coomassie Brilliant Blue-stained gel (Figure 5A), we observed the appearance of four new bands following the cross-linking with apparent molecular mass of 120 (faint band), 96, 73, and 38 kDa, respectively. On the fluorescent picture (Figure 5B),

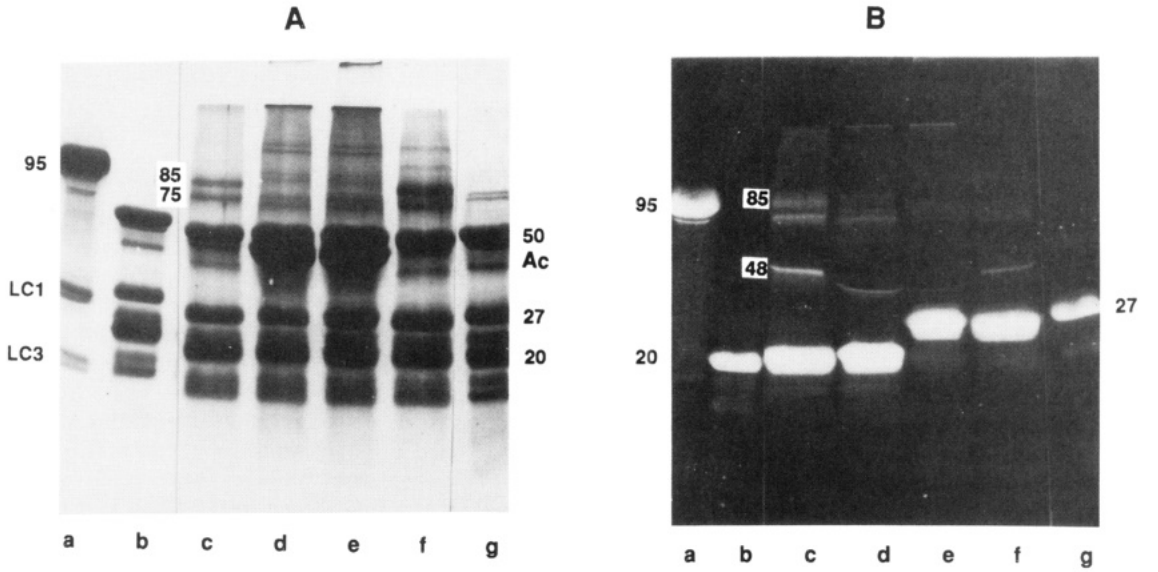

FIGURE 2: Effect of actin on cross-linking of tryptic S1 with EEDQ. S1 was labeled with IAEDANS (lanes a–d) or ANN (lanes e–g): (A) Coomassie Brilliant Blue stained; (B) fluorescent electrophoretogram. Lanes: a, undigested S1; b and g, tryptic S1; c–f, cross-linked tryptic S1; c and f, cross-linking without actin; d and e, cross-linking with actin. Vertical numbers, molecular mass in kilodalton; LC, light chain; Ac, actin.

Table 2: Effect of Cross-Linkers on the ATPase Activity of S1[a]

		ATPase act. in % of untreated control			
cross-linker	MgATP[b]	K^+(EDTA)-activated	Ca^{2+}-activated	Mg^{2+}-activated	actin-activated
EEDQ	–	38	134	83	47
EEDQ	+	12	128	119	36
EDC	–	27	492	173	40
EDC	+	37	415	209	54
glutaraldehyde	–	33	38	151	12
glutaraldehyde	+	45	37	109	4
PDG	–	4	5	18	2
PDG	+	7	8	16	3

[a] For experimental details, see Materials and Methods. [b] MgATP (+) present and (–) absent during the cross-linking.

Table 3: Effect of Cross-Linkers on the Binding of S1 to F-Actin[a]

	S1 bound to F-actin (%)	
cross-linker	50 mM NaCl	100 mM NaCl
none	99.5	98.1
EEDQ	99.1	97.2
EDC	92.3	86.5
glutaraldehyde	42.1	23.5
PDG	98.6	96.9

[a] S1 was cross-linked in the presence of $MgCl_2$. Binding was estimated from cosedimenting S1 with F-actin; for experimental details, see Materials and Methods.

one can observe more bands of cross-linked products, and their composition can be determined by using the fluorescent labels. Accordingly, the 96- and 73-kDa bands are 50-kDa/20-kDa cross-linked products, while the 38-kDa band corresponds to the light chain (A2)/20-kDa product. In addition, there are bands which cannot be seen on the Coomassie Brilliant Blue-stained gel with apparent molecular masses of 74 (Figure 5B, lanes f–h), 46 (Figure 5B, lanes c, d, g, and h), and 42 kDa (Figure 5B, lanes c and d). These correspond to 27-kDa/50-kDa, 27-kDa/20-kDa, and light chain (A1)/20-kDa cross-linked products, respectively. The presence of MgATP was found to only slightly influence the cross-linking. A faint 120-kDa cross-linked band, which contains, among others, the 20-kDa fragment, appears only in the absence of nucleotide (Figure 5A–C, lane c), and the 96- and 38-kDa bands, in some but not all the experiments, are fainter in the

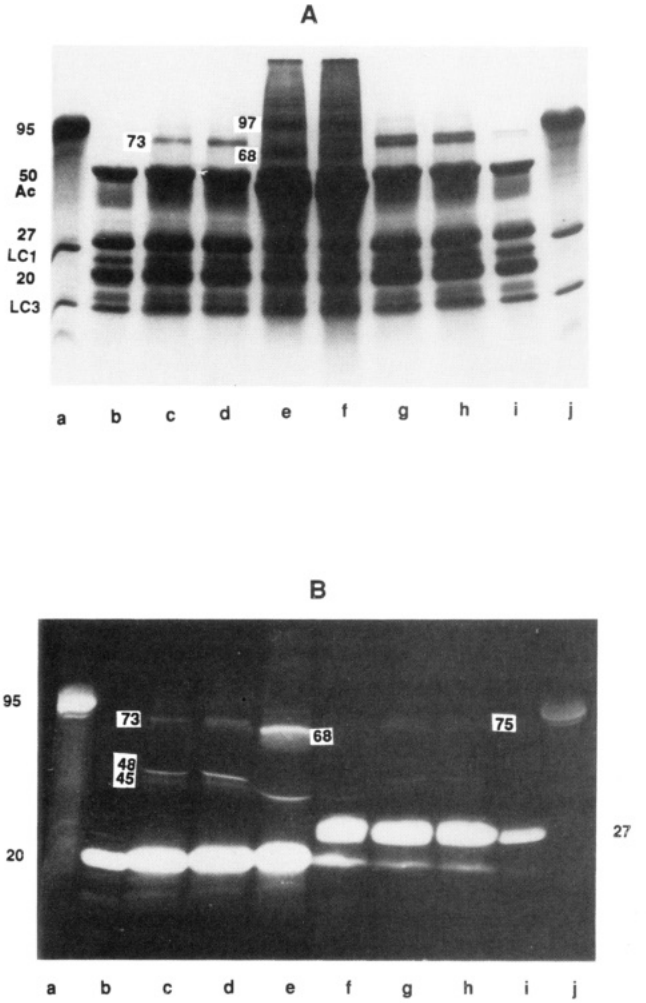

FIGURE 3: Cross-linking of tryptic S1 with EDC. S1 labeled with IAEDANS (lanes a–e) or ANN (lanes f–j). (A) Coomassie Blue stained; (B) fluorescent electrophoretogram. Lanes: a and j, undigested S1; b–i, tryptic S1; c–h, cross-linked tryptic S1; c and f, cross-linking in the presence of MgATP; d and g, cross-linking in the presence of $MgCl_2$; e and f, cross-linking in the presence of $MgCl_2$ and actin. For vertical numbers, see Figure 2.

presence of MgATP than in its absence (Figure 5C, lanes c and d). The effect of actin on the intramolecular cross-link

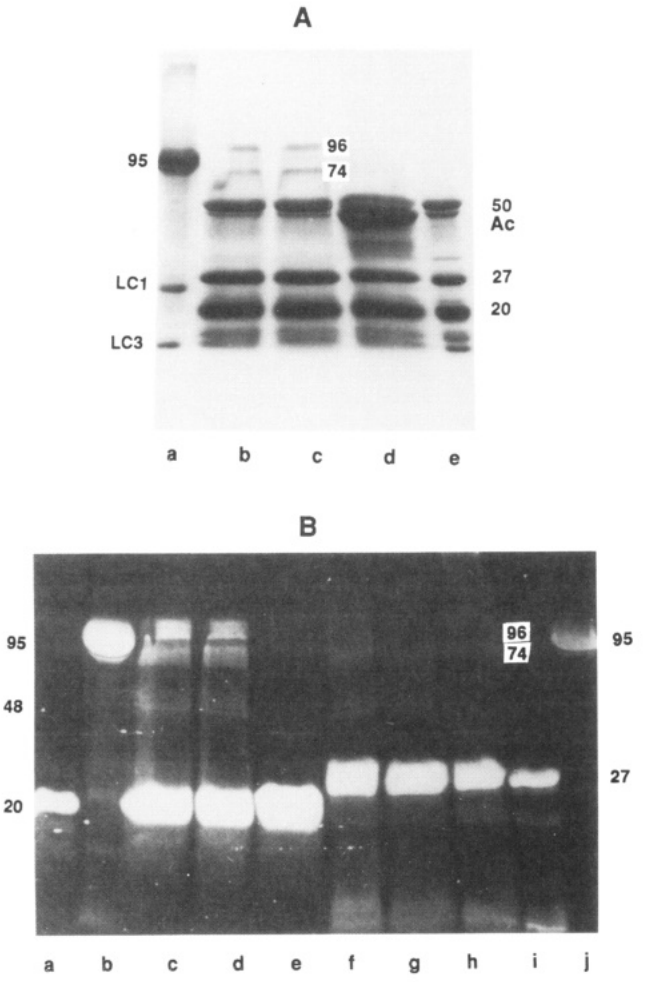

FIGURE 4: Cross-linking of tryptic S1 with glutaraldehyde. S1 (labeled with IAEDANS (lanes a–e) or ANN (panel B, lanes f–j). (Panel A) Coomassie Brilliant Blue-stained gel. Lanes: a, undigested S1; b–e, tryptic S1; b, cross-linking in the presence of MgATP; c, cross-linking in the presence of $MgCl_2$; d, cross-linking in the presence of $MgCl_2$ and actin. (Panel B) Fluorescent electrophoretogram. Lanes: b and j, untreated S1; a and c–i, tryptic S1; c and f, cross-linking in the presence of MgATP; d and g, cross-linking in the presence of $MgCl_2$; e and h, cross-linking in the presence of $MgCl_2$ and actin. For vertical numbers, see Figure 2.

formation is also very minor, only the 96- and 73-kDa bands are stronger in the absence of actin than in its presence (Figure 5A,B).

The various ATPase activities of S1 and tryptic S1 are very strongly affected by the PDG reaction (the results with S1 are shown in Table 2; results with tryptic S1 are similar and not shown). A sharp decrease was observed in all the assayed ATPase activities, and the presence of MgATP only slightly moderated the loss of activity. On the other hand, the binding of S1 to F-actin, as measured in a sedimentation assay, was not influenced by the PDG treatment (Table 3).

DISCUSSION

Intramolecular cross-link formation and nucleotide- and actin-induced intramolecular movements in S1 were comprehensively studied, using four cross-linkers with different specificities. The reaction of these cross-linkers with tryptic S1 results in intramolecular cross-linking. In the cross-linking reactions presented in the Results section, fluorescence labeled tryptic S1 was used, which facilitated the assignment of the fragments participating in the formation of the cross-linked products. In order to check the possible effect of labeling on the cross-linking reactions, we carried out these reactions also with unlabeled tryptic S1 and obtained essentially the same results (not shown), which indicate that the labeling did not influence the cross-linking. The most intensive cross-link formation was observed between the 50- and the 20-kDa heavy chain fragments. With all cross-linkers, two distinct 50-kDa/20-kDa cross-linked products were formed, which could be distinguished from each other on the basis of their electrophoretic mobility. It is a well-known phenomenon that in some cases the electrophoretic mobility of cross-linked peptides is anomalous, generally lower than expected from their molecular mass, and dependent on the position of the cross-link in the sequence. Therefore, two cross-linked species, even if they are products of the same two peptides and have the same molecular mass, can often be separated from each other by electrophoresis if the cross-linked residues have a different sequence location (Sutoh, 1983). The finding that two 50-kDa/20-kDa cross-linked products were formed with a variety of cross-linkers indicates that at least two regions of these fragments are proximal to each other in the S1 structure. On the basis of the recently published atomic structure of S1 (Rayment et al., 1993a), one may speculate which regions of the 50- and 20-kDa fragments were cross-linked to each other. According to a crude estimate (since the coordinates of the individual residues have not been deposited yet), the 647–674 region of the 20-kDa fragment is proximal to the 238–324 stretch of the upper 50-kDa while the 697–709 region is near the 518–529 region of the lower 50-kDa domain, which make these stretches good candidates for cross-linking.

The cross-link formation between the 50- and 20-kDa fragments is affected by both nucleotide and actin. The nucleotide effect was observed with the EEDQ cross-linker in the presence of both MgATP and MgADP, and it manifested itself in more intensive cross-link formation between the two fragments. The increase in intensity was especially conspicuous in the case of the lower electrophoretic mobility (85-kDa) band. The results indicate that two specific regions in the 20- and 50-kDa fragments moved nearer to each other as a consequence of nucleotide-induced conformational changes in S1, and in this respect, no difference was seen between the structural distortions induced by MgADP or MgATP. The fact that the nucleotide effect on the formation of the 50-kDa/20-kDa cross-linked product was observed in our hands only with EEDQ may indicate that only limited and specific regions of the two fragments move toward each other upon the addition of the nucleotide. These conclusions are supported by several former studies. Thus (a) Lu et al. (1986) and Sutoh and Lu (1987) using 4-(2-iodoacetamido)benzophenone cross-linker observed cross-link formation between the SH_1 thiol and a region on the 50-kDa fragment, located 57–60 kDa from the N-terminus of the S1 heavy chain, only in the presence of MgADP; (b) Hiratsuka (1988) reported that MgATP accelerated the cross-linking of the SH_1 thiol to the 50-kDa fragment by methyl bis(2-chloroethyl)amine; and (c) Rajasekharan et al. (1989) and Agarwal et al. (1991) found that MgADP accelerated the cross-link formation with 4-(*N*-maleimido)benzophenone between SH_1 and a region of the 50-kDa fragment located 55–65 kDa from the N-terminus of S1. Also the results of Wakabayashi et al. (1992), using small-angle X-ray scattering, and those of Highsmith (1992), measuring electric birefrigence, indicated nucleotide-induced conformational changes in S1. On the other hand, there are studies which have not reported any effect of nucleotides on the cross-link formation between the SH_1 thiol of the 20-kDa fragment and various residues on the 50-kDa segment. These include reports about cross-link formation between SH_1 and Cys-522 (Ue, 1987) and between SH_1 and residues 485–493 on the 50-kDa fragment (Muno & Sekine, 1988). All these

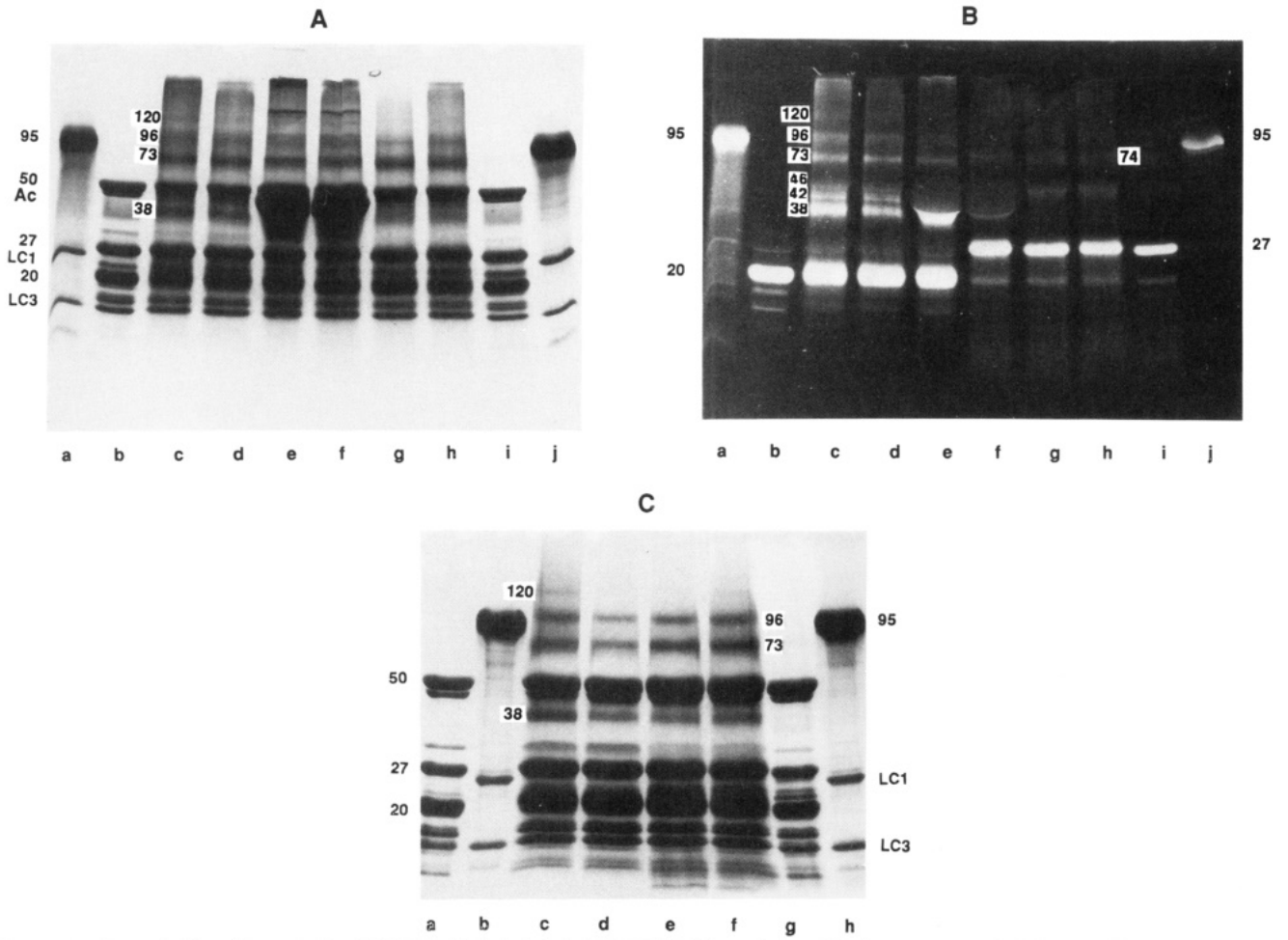

FIGURE 5: Cross-linking of tryptic S1 with PDG. S1 labeled with IAEDANS (panels A and B, lanes a–e; panel C, lanes a–d) or ANN (panels A and B, lanes e–j; panel C, lanes e–h). (Panel A) Coomassie Brilliant Blue-stained gel; (Panel B) Fluorescent gel. Lanes: a and j, undigested S1; b–i, tryptic S1; c–h, cross-linked tryptic S1; c and h, cross-linking in the presence of $MgCl_2$; d and g, cross-linking in the presence of MgATP; e and f, cross-linking in the presence of $MgCl_2$ and actin. (Panel C) Coomassie Brilliant Blue-stained gel. Lanes: b and h, undigested S1; a and c–g, tryptic S1; c–g, cross-linked tryptic S1; c and f, cross-linking in the presence of $MgCl_2$; d and e, cross-linking in the presence of MgATP. For vertical numbers, see Figure 1.

results, together with those which showed that upon addition of nucleotides to S1 there occurs no change in the NMR (Highsmith et al., 1979) or a very slight change in the CD spectrum (Johnson et al., 1991), indicate that the nucleotide-induced movements in the S1 structure are not extended to the whole molecule. On the other hand, the actin-induced structural changes in S1 seem to be more extended, since according to the present results, actin significantly reduces the cross-link formation between the 50- and 20-kDa fragments with all four cross-linkers used. Lu et al. (1987) also observed actin inhibition of the cross-link formation between the 50-kDa fragment and the SH_1 thiol on the 20-kDa fragment, using benzophenone iodoacetamide as a cross-linker. Proton NMR studies, which showed a significant decrease in the mobility of the S1 side chains upon the addition of actin but no change upon the addition of nucleotides (Highsmith et al., 1979), also indicate that the changes induced by actin extend to a larger portion of the molecular structure than those induced by nucleotides. In addition, nucleotides generally increase the cross-link formation between the 20- and 50-kDa fragments, while actin decreases it. The opposing influence of actin and nucleotides on intramolecular cross-linking is in agreement with the assumed transduction function of S1, which predicts reciprocal effects of nucleotides and actin on the S1 structure (Botts et al., 1989), and with the recent hypothesis of Rayment et al. (1993b) on the interaction of myosin with actin and ATP, which is based on the atomic structure of two proteins.

Cross-link formation was observed between the 27-kDa N-terminal and the 20-kDa C-terminal fragments of the S1 heavy chain in the course of the reaction with the four cross-linkers used in our experiments. These products could be seen as faint bands on the fluorescent pictures of the IAEDANS- and ANN-labeled tryptic S1 electrophoretograms but did not appear on the Coomassie Brilliant Blue-stained gels, because these bands overlap with the band of the 50-kDa fragment. The formation of the 27-kDa/20-kDa products in the reactions with the four cross-linkers used in our study was not affected by MgATP. Similarly, no significant nucleotide effect was seen on the cross-linking of the SH_1 thiol with benzophenone iodoacetamide to Glu-88 (Lu et al., 1986; Lu & Wong, 1989) or with 4-(*N*-maleimido)benzophenone to a region at 14–16 kDa from the N-terminus on the 27-kDa fragment (Rajasekharan et al., 1989). Actin inhibits the formation of the 27-kDa/20-kDa cross-link with EEDQ, but has no effect with the three other cross-linkers used in this study. Neither did actin influence the 27-kDa/20-kDa cross-linking in the studies of Lu et al. (1987) with benzophenone iodoacetamide, which couples SH_1 to Glu-88 (Lu & Wang, 1989). In conclusion, the 27- and 20-kDa fragments, which are far away from each other in the S1 primary sequence, are at least partially proximal in the spatial structure of the molecule. This proximity has also been shown by the recently described atomic structure of S1 (Rayment et al., 1993a). The atomic structure indicates that the 714–767 stretch of the 20-kDa fragment is proximal to the 116–126 region of the

27-kDa fragment in addition to the regions described earlier by Lu and Wong (1989) and Rajasekharan et al. (1989), which makes the cross-linking of these regions possible. According to our studies, the interface between the two domains is much less affected by nucleotide- or actin-induced intramolecular movements than that of the 50-kDa/20-kDa interface.

The present study shows for the first time the formation of cross-links between the 27- and 50-kDa fragments. These cross-linked products cannot be seen as separate bands on the Coomassie Brilliant Blue-stained gels, because they partially overlap with the much stronger 50-kDa/20-kDa products; however, they can be recognized as faint bands on the basis of their ANN fluorescence. The 27-kDa/50-kDa product appears as a single band on the electrophoretogram with EEDQ and PDG and as a doublet with EDC and glutaraldehyde as cross-linkers. The latter findings indicate the existence of an extended interface between the two neighboring domains. MgATP did not influence the cross-linking between the 27- and 50-kDa fragments in our experiments. On the other hand, actin inhibits the formation of the 27-kDa/50-kDa products with both EEDQ and EDC, again indicating that the actin-induced conformational changes extend to a larger portion of the S1 molecule than those induced by nucleotides. According to the atomic structure, the interface between the 27- and 50-kDa fragments are quite extended (Rayment et al., 1993a). The 80–90 stretch of the 27-kDa fragment is proximal to the lower 50-kDa domain, and both the 131–204 region of the 27-kDa domain and the 218–324 region of the upper 50-kDa domain participate in the formation of the active site. Therefore, these regions can also be cross-linked to each other.

Cross-link formation between the 20-kDa fragment of the S1 heavy chain and the light chains was also observed in this work using EEDQ, EDC, and PDG as cross-linkers. Both the LC1 and LC3 light chains form cross-link with 20-kDa; however, it was difficult to separate the 20-kDa/LC1 and 20-kDa/LC3 products by SDS–PAGE due to the degradation of the LC1 light chain during the tryptic digestion. Actin inhibits the formation of the 20-kDa/light chain products when either EEDQ or EDC was used, while MgATP inhibition was observed with PDG as the cross-linker. This is the first time that 20-kDa/light chain cross-linked products were directly observed. Similarly, the actin or nucleotide perturbation of the heavy chain/alkali light chain interface has not been reported earlier. The atomic structure of S1 (Rayment et al., 1993a) implies that the formation of cross-links between the 20-kDa fragment and the essential light chains (LC1 and LC2) can take place because the light chain is wrapping up the 777–814 helical stretch of the 20-kDa fragment.

We have also studied the effect of cross-linking on the ATPase activity of both S1 and tryptic S1. In these studies, unmodified protein was used in order to avoid the effect of IAEDANS and ANN labeling on the ATPase activity. The reaction with all four cross-linkers profoundly affected the various ATPase activities. The magnitude of the effect was generally so great that it cannot be attributed only to the cross-linking process—because a relatively small fraction of S1 has become cross-linked during the reactions—but to the modification of various residues by the cross-linkers (only a fraction of the residues, which are modified in the first step of the cross-linking reaction, are actually cross-linked to another residue in the second step because of the lack of a reactive residue in the proximity). Both actin-activated and K^+(EDTA)-activated ATPase activities sharply decreased as a consequence of the reaction with all four cross-linkers. The change in Ca^{2+}-activated ATPase depends on the cross-linker used: a very strong increase was observed with EDC, less increase with EEDQ, and a decrease with PDG and glutaraldehyde. The more than 4-fold increase in Ca^{2+}-ATPase together with the loss in K^+(EDTA)-ATPase in the case of EDC treatment resembles the effect of SH_1 modification on myosin ATPase (Sekine & Kielley, 1964). In spite of the fact that EDC specifically activates carboxyl groups, one cannot a priori exclude the possibility of the reaction of EDC with the reactive SH_1 thiol. The Mg^{2+}-modulated ATPase activity was found to increase after the treatment with all the reagents with the exception of PDG, which caused a decrease. After treatment with this arginine-specific reagent, all ATPase activities were strongly inhibited in agreement with the results of Bonet et al. (1988). The strong inhibition points to the role of some unidentified arginine residues in the ATPase activity of myosin (Mornet et al., 1979b). The effect of glutaraldehyde on the various ATPase activities of S1 in our experiments was similar to those observed by Hiratsuka (1986), who also studied the cross-linking of tryptic S1 with this reagent. We found that the presence of nucleotide during the reaction has generally a moderating influence on the changes in the ATPase activities, except in the case of EEDQ which caused larger alterations in the presence of ATP than in its absence. This latter finding is in accord with the observed increase in the 50-kDa/20-kDa cross-linking with EEDQ in the presence of nucleotides.

Finally, we assessed the effect of the various cross-linking reagents on the S1–actin interaction in a sedimentation assay under rigor conditions—using in these studies unlabeled S1. The treatment with EEDQ and PDG did not affect the interaction while the treatment with EDC and glutaraldehyde, moderately and severely inhibited the binding of S1 to F-actin, respectively. This latter finding is in accord with the almost complete loss of actin-activated ATPase activity observed upon reaction of S1 with glutaraldehyde. It seems that glutaraldehyde reacts with those amino groups which are essential for the actin–S1 interaction in both the rigor and weakly attached states.

In conclusion, cross-linking studies can facilitate the description of the S1 architecture and the nucleotide- and actin-induced intramolecular movements. We found that nucleotide-induced intramolecular movements are mainly confined to specific regions of the 50-kDa/20-kDa and 20-kDa/alkali light chain interfaces, while the actin-induced movements are less localized and extend to large regions of the S1 segment of myosin.

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