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Interaction of the Heavy Chain of Scallop Myosin Heads with Skeletal F-Actin†

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ABSTRACT: The Ca^{2+}-regulated molluscan myosin from scallop muscle and its subfragment 1 (+LC) were cross-linked to skeletal and scallop F-actins by using 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDC) under conditions avoiding protein aggregation. The elevated Mg^{2+}-ATPase of the covalent acto-S-1 was as high in the absence as in the presence of Ca^{2+}. In contrast, the cross-linking of the intact myosin to actin led to an enhancement of the Mg^{2+}-ATPase that was 2-fold higher in the presence than in the absence of Ca^{2+}. Calcium had no effect on the stimulated ATPase of the covalent complex formed with desensitized myosin. Cross-linked adducts between actin and the 31K or 24K heavy chain peptides present in the tryptic (63K–31K)-S-1 and (70K–24K)-S-1 were produced but not between actin and the corresponding N-terminal segments. Joining the former derivative to actin led to an inactive covalent complex, indicating that an irreversible Mg^{2+}-ATPase loss follows the production of this split S-1 [Szentkiralyi, E. M. (1987) *J. Muscle Res. Cell Motil. 8*, 349–357]. We further show that this ATPase inhibition is specifically associated with the heavy chain scission at the 63K–31K site as proteolysis within the 31K–24K difference segment by *Staphylococcus aureus* protease did not impair the S-1 enzymatic activities. The 17-residue N-terminal sequence we determined for the isolated 31K peptide was not shared by myosins from vertebrate striated muscles. It would reflect the observed unique proteolytic behavior of the scallop S-1 heavy chain and a particular structural relationship between the 63K–31K junction and the ATPase site. Finally, the isolated, renatured, soluble 24K fragment failed to interact with actin, in contrast with the homologous peptide from skeletal S-1. Conversly, actin did not bind to a new trypsin S-1 derivative formed in the presence of Mg^{2+}-ATP and having an intact N-terminal 68K segment but a degraded 24K moiety. These data imply that critical interactions between the associated NH_2- and COOH-terminal regions of the S-1 heavy chain are required for the specific attachment of actin to the scallop myosin head.

The contraction of molluscan striated muscles depends on the force-generating interactions between the actomyosin complex and Mg^{2+}-ATP in the presence of calcium. The specific binding of Ca^{2+} ions to a regulatory subunit of myosin (Szent-Gyorgyi et al., 1973; Chantler & Szent-Gyorgyi, 1980) induces a structural rearrangement of a vicinal essential light chain (Hardwicke & Szent-Gyorgyi, 1985) that alters the ATPase site conformation and results in the activation of the actomyosin Mg^{2+}-ATPase through acceleration by actin of the

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rate of a kinetic step of ATP hydrolysis (Chalovich et al., 1984; Wells & Bagshaw, 1985). In the absence of calcium the binding of actin to the myosin heads has no significant effect on the Mg^{2+}-ATPase activity.

Knowledge of the molecular mechanisms of energy transduction in invertebrate muscles would require the understanding of the structural relationships between the ATPase and actin sites on the myosin head heavy chain. The nature and location of the actin recognition sites on the molluscan myosin subfragment 1 (S-1)[1] are as yet unknown. However, recent studies using limited tryptic proteolysis of scallop S-1 and acto-S-1 have suggested common properties of scallop and rabbit skeletal S-1s in regard to actin interaction (Szentkiralyi, 1984). Thus, actin binding protects the scallop S-1 (+LC) heavy chain against its tryptic cleavage into 73K (NH_2-terminal) and 24K (COOH-terminal) fragments, as it protects the rabbit S-1 heavy chain against splitting at the protease-sensitive 70K–20K junction (Mornet et al., 1979a; Chaussepied et al., 1983); also, for both S-1s, this scission of the heavy chain results in the abolition of the actin-activated ATPase activity. In addition, the controlled tryptic digestion of the scallop acto-S-1 complex results in the production of a relatively stable (63K–31K)-S-1 entity with concomitant loss of the Ca^{2+}- and K^+-dependent ATPase activities (Szentkiralyi, 1984, 1985, 1987). This derivative is probably homologous to the inactive thrombin-cut (68K–30K)-S-1 recently obtained from the skeletal thiol-modified chymotryptic S-1 by peptide bond cleavage at Lys (560) (Chausepied et al., 1986a,b). Their similar enzymatic behavior could reflect the property of the NH_2-terminal 7K–10K portion of the 30K carboxyl-terminal heavy chain segment to communicate with both the ATPase and actin sites; this is also suggested by the ability of the isolated skeletal 30K peptide to undergo a reversible, ATP-dependent association with F-actin (Chaussepied et al., 1986c).

In the present study, we have employed the carbodiimide-catalyzed chemical cross-linking between actin and myosin (Mornet, 1981b) as well as the nucleotide-dependent proteolysis of S-1 (Mornet et al., 1985) to assess further the interaction of rabbit skeletal F-actin with the regulated scallop myosin and with the unregulated scallop papain S-1 (+LC) and its various proteolytic derivatives. The results describe for the first time the direct cross-linking of the intact scallop myosin to F-actin and the involvement of the COOH-terminal 24K segment of the S-1 heavy chain as the unique site for this covalent union and indicate also that the native conformation of the 24K domain within the S-1 heavy chain is required for the tight attachment of actin to the scallop myosin head. Moreover, we have isolated the longer tryptic 31K fragment from the scallop ATPase inactive (63K–31K)-S-1, and we have characterized this peptide by NH_2-terminal amino acid microsequencing.

Materials and Methods

Chemicals. Trypsin treated with L-1-(tosylamido)-2-phenylethyl chloromethyl ketone was obtained from Worthington Biochemicals. *Staphylococcus aureus* V8 protease was purchased from Miles, and 1-ethyl-3-[3-(dimethylamino)-propyl]carbodiimide was from Sigma. All other chemicals were of the highest analytical grade.

Preparation of Proteins. Myosin was prepared from striated adductor muscle of the scallop (*Pecten maximus*) by the method of Stafford et al. (1979). It was stored at −30 °C in 50% glycerol for no more than 1 month.

Desensitized myosin was obtained by the procedure of Wallimann and Szent-Györgyi (1981). Regulatory light chains (R-LCs) were removed by a 5-min exposure to 10 mM EDTA in 40 mM NaCl and 5 mM phosphate buffer, pH 7.0 at 23 °C. Myosin was pelleted by centrifugation and washed twice with 40 mM NaCl and 5 mM phosphate buffer, pH 7.0.

Scallop S-1 (+LC) was isolated from myosin (10 mg/mL) by papain-activated digestion (0.05 unit of papain/mg of myosin) for 10 min in 20 mM phosphate buffer and 100 mM NaCl, pH 7.0, in the presence of 3 mM $MgCl_2$. To stop the reaction, the digest was incubated with 1 mM iodoacetic acid for 5 min at room temperature followed by the addition of dithiothreitol to 5 mM. The digestion mixture was dialyzed overnight against 10 mM phosphate, pH 7.0, and centrifuged at 170000*g*, 45 min, 4 °C; the solution was brought to a final protein concentration of 10–20 mg/mL by ultrafiltration on an Amicon PM10 membrane.

Rabbit chymotryptic myosin S-1 was obtained according to the method of Weeds and Taylor (1975). The scallop split-S-1 derivatives (63K–31K)-S-1 and (70K–24K)-S-1 were prepared by limited tryptic proteolysis of native scallop S-1 (+LC) in the presence and absence of F-actin, respectively, essentially as described by Szentkiralyi (1984) using 50 mM imidazole–HCl buffer, pH 7.0, and molar ratio F-actin:S-1 of 2. Similar conditions were employed for the tryptic digestion of the isolated EDC covalent acto-S-1 complex and for the splitting of S-1 in the presence of nucleotides.

The scallop 24K peptide light chains complex was isolated essentially following the procedure of Chaussepied et al. (1986d).

The digestions of S-1 (5 mg/mL) with staphylococcal protease was carried out at a weight ratio of protease to S-1 of 1:100 in 50 mM imidazole–HCl, pH 7.5 at 20 °C.

Rabbit skeletal and scallop F-actins were purified according to the method of Eisenberg and Kielly (1974).

F-Actin and S-1 were labeled with the fluorescent dye *N*-(iodoacetyl)-*N'*-(5-sulfo-1-naphthyl)ethylenediamine (1,5-IAEDANS) as reported by Mornet et al. (1981a) and Wells and Bagshaw (1983), respectively.

Protein concentrations were determinated according to the method of Wells and Bagshaw (1983).

ATPase Activities. The Mg^{2+}- and actin-activated Mg^{2+}-dependent ATPase activities were measured as specified earlier (Mornet et al., 1979b). ATPase assays of the covalent complexes were conducted for 20 min with 0.050 mg/mL myosin or S-1 and 0.050 mg/mL F-actin. Measurements of the Mg^{2+}-ATPase of myosin were carried out with 1 mg/mL protein for 20 min.

Cross-Linking of F-Actin–Myosin and F-Actin–S-1 Complexes with Carbodiimide. F-Actin (2 mg/mL) and scallop myosin (2 mg/mL) were combined in 1 mL of 50 mM imidazole buffer, pH 7.0. Cross-linking experiments were performed for 0–60 min at room temperature in the presence of 1 mM EDC added from a freshly prepared stock solution (15 mM in water).

To obtain covalent complexes between F-actin (2 mg/mL) and S-1 or S-1 derivatives (2 mg/mL), the EDC-activated

[1] Abbreviations: ATPase, adenosine-5'-triphosphatase (EC 3.6.1.3); S-1, myosin subfragment 1; Acto-S-1, actomyosin S-1; R-LC, scallop myosin regulatory light chain; mR-LC, papain split regulatory light chain; SH-LC, scallop myosin essential light chain; LC1 and LC3, vertebrate myosin alkali light chain 1 and 3; EDC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide; IAEDANS, *N*-(iodoacetyl)-*N'*-(5-sulfo-1-naphthyl)ethylenediamine; $NaDodSO_4$, sodium dodecyl sulfate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N'*,*N'*-tetraacetic acid; TFA, trifluoroacetic acid; HPLC, high-performance liquid chromatography.

F-actin (Mornet et al., 1981b) was mixed at room temperature with S-1 in 50 mM imidazole, buffer pH 7.0.

The cross-linked acto-S-1 complexes formed after a 10-min reaction were isolated by centrifugation at 170000*g* for 1 h at 4 °C, after dissociation of un-cross-linked S-1 with 10 mM sodium pyrophosphate and 5 mM $MgCl_2$ in 50 mM imidazole buffer, pH 7.0.

Sodium Dodecyl Sulfate Gel Electrophoresis. The cross-linked protein species were separated by electrophoresis in 0.1% $NaDodSO_4$ with 4.5–17.5% gradient polyacrylamide gels using a 50 mM Tris–100 mM boric acid buffer, pH 8.0, as described by Mornet et al. (1981a).

The chain weights of protein entities were determined with the following molecular weight markers: skeletal chymotryptic S-1 heavy chain (95K), actin (42K) and carbodiimide cross-linked actin-skeletal S-1 heavy chain (160K); skeletal trypsin split (27K–50K–20K)-S-1 and thrombin-cut (68K–30K)-S-1 (Chaussepied et al., 1986a). Gels were stained with Coomassie brillant blue R-250 and destained according to the method of Weber and Osborn (1969). The gels were scanned with a Shimadzu Model CS930 high-resolution gel scanner equipped with a computerized integrator. Proteolytic COOH-terminal fragments of the scallop S-1 heavy chain were also monitored on gels by a nitrocellulose overlay technique using 1,5-IAEDANS-labeled F-actin (Burnette, 1981; Suzuki & Morita, 1987). Fluorescent protein bands were located in the gels by illumination with long-wave ultraviolet light before they were stained with Coomassie blue.

Binding Experiments. The association of actin with S-1 or the proteolytic S-1 derivatives in the absence and presence of 5 mM Mg^{2+}-ATP was analyzed by sedimentation in a Beckman airfuge essentially as described by Chalovich and Eisenberg (1982) and Chaussepied et al. (1986b).

Peptide Purification and Amino Acid Sequence Analysis. A 6-min tryptic digest of the scallop S-1–actin complex was brought to 10 mM magnesium pyrophosphate and centrifuged at 70000*g* for 1 h at 4 °C. The supernatant was then dialyzed overnight against 2 mM sodium phosphate, pH 7.5. An aliquot of the digested S-1 solution (1 mg) was adjusted to pH 2.5 with 1 M TFA and then submitted to reverse-phase HPLC using a Waters Associates gradient liquid chromatograph and a 4.6 mm × 75 mm Beckman Ultrapore RPSC column. A linear gradient elution was performed in 60 min using solvent A (0.1% TFA in water) and solvent B (0.1% TFA in 60% acetonitrile). The fractions containing the 31K heavy chain peptide were further purified on the same column using two successive linear gradient solutions consisting respectively of 0–33% acetonitrile in 0.1% TFA (5-min elution) and 33–60% acetonirile in 0.1% TFA (55-min elution).

Amino acid sequences were determined on an Applied Biosystems Model 470A gas-phase protein sequencer operated as described by Hewick et al. (1981), and PTH amino acids were analyzed by reverse-phase HPLC using an Applied Biosystems Model 120A analyzer.

RESULTS

Cross-Linking of F-Actin to Scallop Myosin and Its Subfragment 1. We first investigated the cross-linking of the regulated scallop myosin to skeletal F-actin by the carbodiimide reagent (Mornet et al., 1981b) under selected optimal experimental conditions that minimize protein aggregation (pH 7.0, 25 °C, 1 mM EDC, low ionic strength, filamentous myosin). The covalent union between actin and myosin was followed by measuring the changes in the Mg^{2+}-ATPase activity as assayed in the absence and presence of calcium. The results are presented in Figure 1A. In both cases the EDC

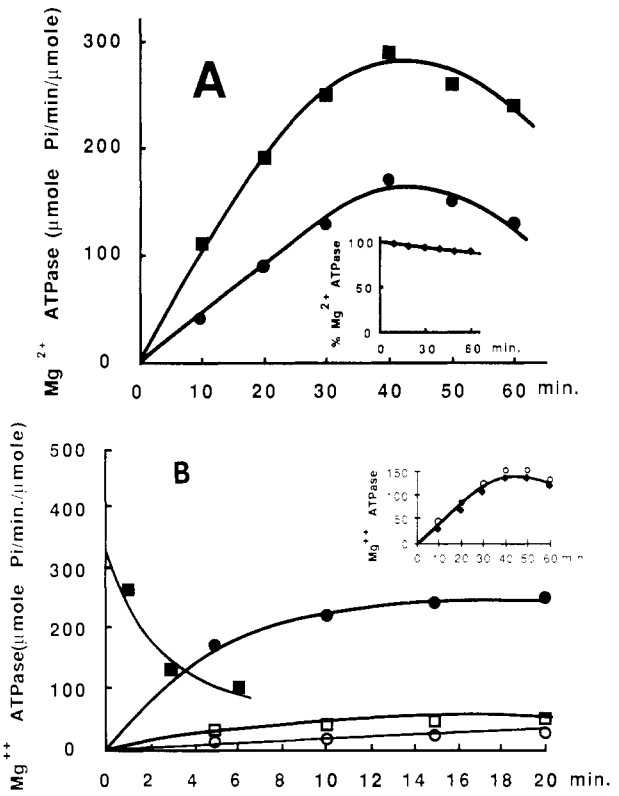

FIGURE 1: Stimulation of Mg^{2+}-ATPase of myosin, S-1, and tryptic S-1 derivatives covalently cross-linked to F-actin. (A) Scallop myosin (2 mg/mL) was incubated in 50 mM imidazole–HCl at pH 7.0 and 20 °C with skeletal F-actin (2 mg/mL) and EDC (1 mM). At the times indicated, aliquots were withdrawn and submitted to Mg^{2+}-ATPase assays in the presence of 0.1 mM EGTA (●) or 0.1 mM Ca^{2+} (■). At zero time, before EDC addition, the actin-activated Mg-ATPase activity was not measurable under the conditions employed, both in the absence and in the presence of Ca^{2+} ions. (Inset) Mg^{2+}-ATPase activity of myosin (μmol of P_i min)$^{-1}$ (μmol of protein)$^{-1}$ after incubation with EDC in the absence of actin. ATPase determinations were carried out as specified under Materials and Methods. (B) Native S-1 (+LC) (●), 3-min trypsin split (63K–31K) S-1, (□) or 6-min trypsin split (63K–31K-S-1 (○) (2 mg/mL) were combined at pH 7.0, 20 °C, with EDC–F-actin (2 mg/mL) under the conditions reported under Materials and Methods. At the times indicated, protein samples were removed and assayed for Mg^{2+}-ATPase activity. The pellet of covalent acto-S-1 (■) was isolated and digested with trypsin in 50 mM imidazole–HCl, pH 7.0 at 20 °C. At the times indicated protein aliquots were analyzed for Mg^{2+}-ATPase activity. (Inset) Desensitized myosin complexed to F-actin was reacted with EDC as in (A). At the times indicated, the Mg^{2+}-ATPase activity was measured in the absence (■) and presence of Ca^{2+} (○).

reaction induced a rapid enhancement of the Mg^{2+}-ATPase; however, for all samples analyzed during the interval time of the cross-linking process (0–60 min), this activity was nearly 2-fold higher when it was determined in the presence of rather than in the absence of Ca^{2+} ions. The maximal extent of the ATPase stimulation was reached after 35–40 min, and it was always followed by a progressive inactivation phase resulting, probably, from slow secondary intramolecular reactions within the actomyosin complex. Similar results were also observed when scallop F-actin was employed (data not shown). The time course and amplitude of the ATPase activation were not affected by the addition of 0.1 mM Ca^{2+} or EGTA in the reaction medium. The original Mg^{2+}-ATPase activity of the myosin was unchanged when the protein was incubated with EDC under identical conditions but in the absence of actin (Figure 1A, inset); also, the electrophoretic pattern of this EDC–myosin control was not different from that of the native protein. These data indicate that the EDC treatment of the scallop myosin under the conditions we employed had no de-

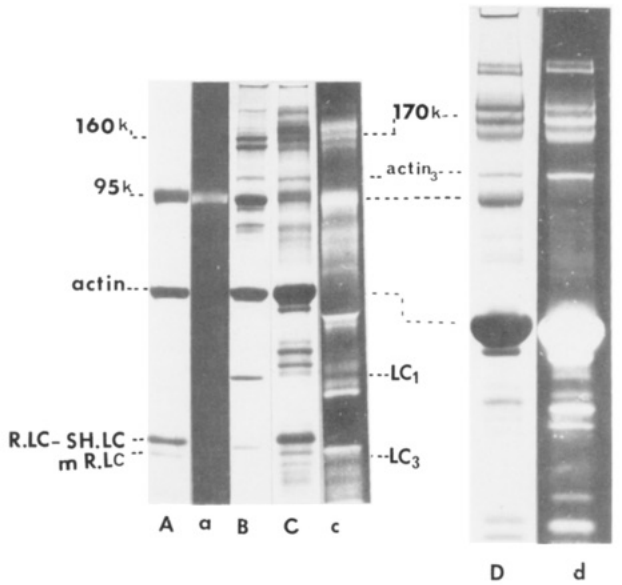

FIGURE 2: $NaDodSO_4$ gel electrophoretic analysis of acto-S-1 cross-linking with EDC. Fluorescent S-1 (+LC) (2 mg/mL) complexed to EDC–F-actin (2 mg/mL) (Cc) and fluorescent F-actin–EDC complexed to S-1 (+LC) (Dd) were incubated in parallel at pH 7.0, 20 °C, for 10 min; protein samples were subjected to 4.5%–17.5% gradient polyacrylamide gel electrophoresis. Cross-linked EDC–actin–skeletal S-1 was run as control (B). Fluorescent scallop S-1–F-actin complex is shown before cross-linking (Aa). Gels were viewed under ultraviolet light (a, c, and d) and then stained with Coomassie blue (A–D).

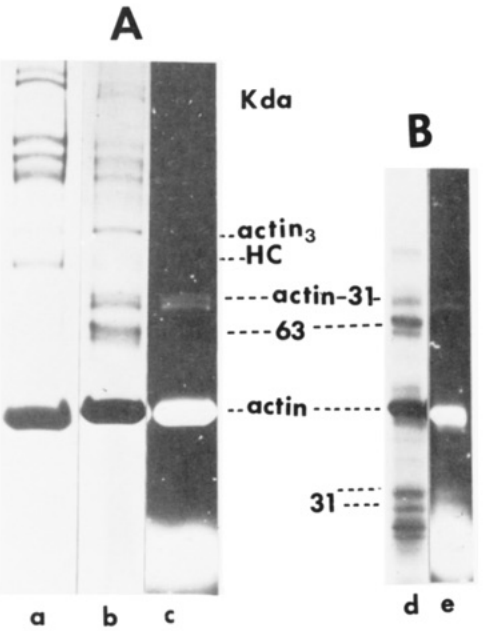

FIGURE 3: Cross-linking of actin to the C-terminal 31K tryptic fragment of the scallop S-1 heavy chain. Fluorescent F-actin was cross-linked with EDC to the preformed tryptic (63K–31K)-S-1 (B, d, and e) under the conditions reported under Materials and Methods. The isolated covalent complex formed between fluorescent actin and native S-1 was also digested with trypsin for 6 min (A, a–c) as indicated under Materials and Methods. The cross-linked species were identified by gradient gel electrophoresis; gels were viewed under UV light (c and e) and then stained with Coomassie blue (a, b, and d).

trimental effects on its structure or function. Consequently, we conclude that the elevated Mg^{2+}-ATPase activity observed in the presence of actin and EDC was dependent on the production of an EDC-catalyzed cross-linked actomyosin complex. On the other hand, the apparent Ca^{2+} sensitivity of the ATPase of this complex is dependent on the presence of the myosin regulatory light chains since, as illustrated in Figure 1B (inset), calcium had no effect on the elevated ATPase activity of the covalent complex formed between actin and desensitized myosin.

To further delineate the interaction of actin with the scallop myosin head heavy chain, scallop papain S-1 and some of its proteolytic derivatives were also cross-linked to actin. Preliminary experiments conducted under reaction condition similar to those previously employed for the skeletal S-1 (Mornet et al., 1981b) indicated that the scallop S-1 is much more prone to aggregation in the presence of EDC; this behavior is certainly related to the reported tendency of native scallop S-1 to undergo self-association (Szentkiralyi, 1984). After several trials and errors, we adopted the optimal experimental conditions that make use of the reaction of EDC-activated actin with S-1 in imidazole buffer, pH 7.0. These were established by monitoring carefully the cross-linking events by using ATPase measurements and gel electrophoretic analyses of the reaction mixtures. Figure 1B illustrates the time course of the vast activation of the Mg^{2}-ATPase of scallop S-1 upon cross-linking to skeletal EDC–actin. This activation plateaued after 15–20 min, without the subsequent inactivation step observed with the parent myosin. The amplitude of the ATPase enhancement was similar in the absence and presence of calcium. In addition, almost no aggregated protein material was present on the top of the electrophoretic gels (Figure 2).

This cross-linking procedure was thereafter applied to the covalent linkage between actin and the split (63K–31K)-S-1 produced by limited digestion of the reversible actin–S-1 (+LC) complex with trypsin (Szenkiralyi, 1984, 1985). The experiments were performed on two fragmented S-1 samples obtained after 3 and 6 min of proteolysis, respectively. Figure 1B shows clearly that in both cases the Mg^{2p}-ATPase stimulation did not occur significantly. The small extent of ATPase activation observed was due to residual uncleaved S-1 as tested by gel electrophoresis (data not shown). This result was expected since the scission of the scallop S-1 heavy chain at the 63K–31K junction was reported to alter the ATPase site with concomitant suppression of the K^+- and Ca^{2+}-dependent ATPases (Szentkiralyi, 1984, 1985). This interpretation was further supported by the extensive loss of super ATPase activity of the preformed covalent acto-S-1 complex upon treatment with trypsin (Figure 1B) and production of the covalent actin–(63K–31K) complex (Figure 3A). Thus, the actual cross-linking of actin to the heavy chain of the nicked (63K–31K)-S-1 species, which we will describe in the next part of this work, did not restore the S-1 ATPase activity. This is in contrast with the large increase of the Mg^{2+}-ATPase activity that takes place on cross-linking actin to the skeletal (27K–50K–20K)-S-1 (Labbé et al., 1982; Mornet & Ue, 1985), a tryptic derivative having a lower affinity for actin in the presence of ATP but an intact ATPase site (Mornet et al., 1979a; Botts et al., 1982).

Identification of the S-1 Heavy Chain Segment Cross-Linked to F-Actin. To characterize the regions of the scallop S-1 heavy chain in close contact with F-actin, the cross-linking experiments were carried out on rigor complexes including fluorescent, IAEDANS-labeled S-1, or F-actin (Mornet et al., 1981b). After dissociation of most of the unreacted S-1 molecules with magnesium pyrophosphate, the covalent acto-S-1 complex was isolated by centrifugation, and the cross-linked species formed were analyzed by electrophoresis on a 4.5–18% gradient gel. The results are illustrated in Figure 2. The main reaction product was a sharply defined triplet band with an apparent mass in the vicinity of 170K (Figure 2, lanes C and D). Its mobility was close to that of the major 160K protein doublet present in the skeletal EDC-cross-linked

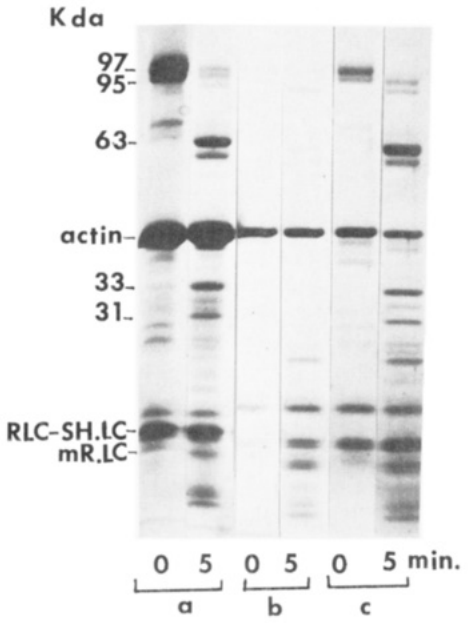

FIGURE 4: Reversible binding of actin to (63K–31K)-S-1. Native S-1 (+LC) combined to F-actin was digested with trypsin for 5 min (a) as indicated under Materials and Methods. After of the reaction was quenched with soybean trypsin inhibitor, the digest was centrifuged in a Beckman airfuge in the absence and presence of 5 mM Mg^{2+}-ATP; the corresponding supernatants (b and c, respectively) were analyzed by gradient gel electrophoresis. The acto-S-1 complex at zero time of digestion was sedimented similarly and used as control.

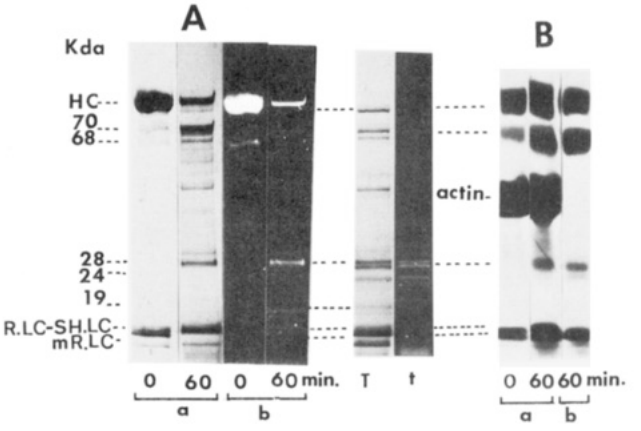

FIGURE 5: Limited proteolysis of S-1 and acto-S-1 with staphyloccocal protease. (A) IAEDANS-labeled S-1 (+LC) (5 mg/mL) in 50 mM imidazole buffer, pH 7.5, was treated with *S. aureus* V8 protease (1:100 w/w) at 20 °C. At the times indicated, the reaction was quenched by mixing 25-μL aliquots of the digest with 175 μL of boiling 5% β-mercaptoethanol and 4% $NaDodSO_4$ solution, and 100-μg protein samples were analyzed by 4.5%–18% gradient gel electrophoresis. The gel pattern after staining with Coomassie blue (a) is compared with the corresponding fluorescence pattern (b). An 8-min trypsin digest of fluorescent S-1 (T and t) was run as control. (B) Time course of digestion of native S-1 (+LC) in the presence (a) and absence (b) of F-actin (actin:S-1 molar ratio = 2) under the conditions described in (A).

acto-S-1 complex used as control (Figure 2, lane B). It incorporated the fluorescence of both S-1 and actin (Figure 2, lanes c and d) and is likely to represent the major cross-linked adduct of actin and the scallop S-1 heavy chain. As observed with the covalent actin–S-1 complex from skeletal myosin, the cross-linked scallop acto-S-1 preparation displayed also a minor 240K protein doublet that contained the fluorescence of either actin or S-1. No protein bands accounting for the cross-linking of the light chains to actin or the heavy chain were detected.

When the isolated, cross-linked IAEDANS-F-actin–S-1 complex was submitted to digestion with trypsin (Figure 3A, lanes a–c), which cuts the actin-bound heavy chain at the 63K–31K junction, the band intensity of the 170K triplet markedly decreased with the concomitant production of two new peptide bands. One had M_r of 72K and contained the actin fluorescence; the other with M_r of 63K was not fluorescent. The former species tended to migrate on the gel as a doublet. We attributed this product to the cross-linking between an actin subunit and the COOH-terminal 31K segment of the S-1 heavy chain. The nonfluorescent 63K entity could be only the NH_2-terminal counterpart of the cross-linked heavy chain; it was not at all engaged in the cross-linking with actin and, therefore, it was released by trypsin in the free state. Our assignments were confirmed by the data obtained on cross-linking fluorescent F-actin to the preformed, enzymatically inactive (63K–31K)-S-1 derivative (Figure 3B, lanes d and e). Ultracentrifugation experiments showed this nicked S-1 to interact reversibly with actin (Figure 4). The reaction led to the fluorescent actin–31K doublet only, whereas the NH_2-terminal 63K fragment remained non-cross-linked to actin, displaying a velocity similar to that of the 63K band deriving directly from the digestion of the intact covalent acto-S-1 complex. Finally, fluorescent actin was also cross-linked to the (70K–24K)-S-1 derivative, which forms predominantly upon a short tryptic digestion of S-1 (+LC) in the absence of actin. Although this nicked S-1 has no actin-activated ATPase activity (Szentkiralyi, 1984), it combines reversibly with actin as assessed by ultracentrifugation of the complex in the absence and presence of 5 mM Mg^{2+}-ATP. The cross-linking reaction led to the formation of a population of 2–3 fluorescent 62K bands that could be the adducts of actin and the 24K peptide as well as of actin and some degraded forms of the latter peptide (data not shown). On the other hand, no fluorescent actin–70K peptide material migrating at the 110K position was noticed.

Selective Cleavage of the 31K–24K Difference Segment with Staphyloccocal Protease. To more precisely locate the ATPase inhibitory clip site within the (63K–31K)-S-1, we investigated the digestion of the native S-1 (LC+) with *S. aureus* V8 protease and its influence on the enzyme ATPase activities. As illustrated in Figure 5A, this protease converted the intact S-1 heavy chain into a 68K–28K derivative without apparently affecting the light chains. The fluorescence of IAEDANS-labeled S-1 was associated mainly with the 28K fragment, which must represent the COOH-terminal heavy chain segment containing the reactive thiols of scallop S-1 (Bennet et al., 1984; Titus & Szent-Gyorgyi, 1986). Thus the *S. aureus* protease attack would occur in just a region within the vulnerable 31K–24K difference segment where the trypsin cuts reside. The binding of F-actin to S-1 decreased the rate of the heavy chain cleavage without changing the proteolytic pattern (Figure 5B). Most importantly, ATPases measurements conducted during the entire course of the *S. aureus* protease reaction indicated no significant alteration of the K^+,Ca^{2+}- and actin-stimulated ATPase activities (data not shown). We conclude that these functions require more specifically the structural integrity of the trypsin-sensitive 63K–31K and 70K–24K junctions than that of the overall 31K–24K difference fragment of the S-1 heavy chain.

Identification of the Amino-Terminal Structure of the Tryptic 31K Fragment. A direct structural characterization of the ATPase-related 63K–31K site was further accomplished by the isolated of the 31K peptide and determination of its NH_2-terminal amino acid sequence. We first monitored the time course of the production of the 31K entity from the trypsin-treated acto-S-1 under our experimental conditions,

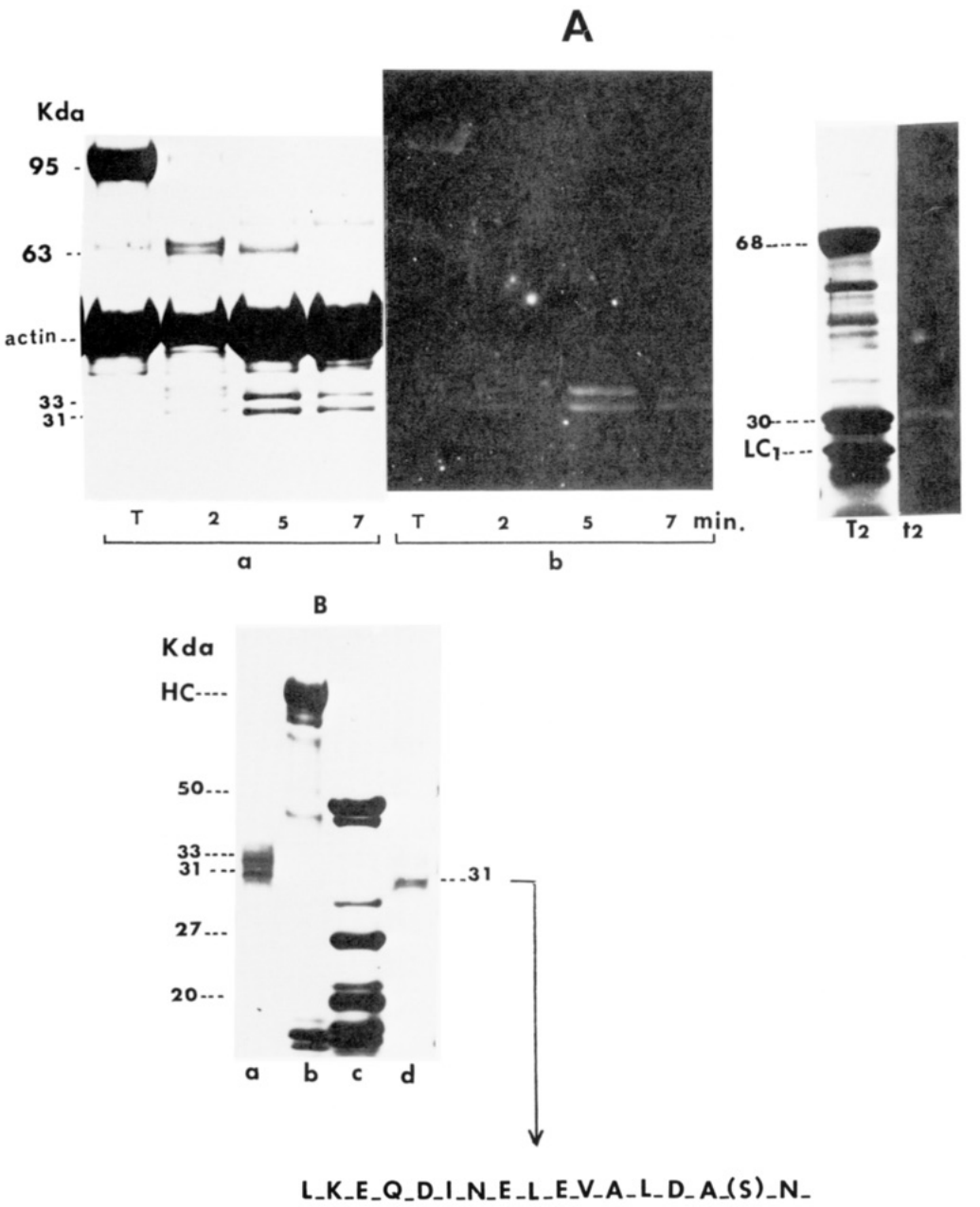

FIGURE 6: Isolation and NH_2-terminal sequence of the 31K C-terminal fragment of the S-1 heavy chain. (A) S-1 (+LC) combined to F-actin (both at 2 mg/mL) was digested with trypsin (protease:S-1 weight ratio = 1:100) in 50 mM imidazole–HCl, pH 7.0 at 20 °C. At the times indicated the fragmentation pattern was established in duplicates by gradient gel electrophoresis. The protein bands were stained with Coomassie blue (a) or transferred to a nitrocellulose sheet by electroblotting and overlayed by fluorescent, IAEDANS-labeled F-actin (b). (T) is acto-S-1 before digestion; control skeletal thrombic (68K–30K)-S-1 (A1 + A2) was stained with Coomassie blue (T2) and with fluorescent actin (t2). (B) the 31K peptide containing fractions obtained after the first (a) and second (d) HPLC were identified by gradient gel electrophoresis. Native scallop S-1 (b) and skeletal trypsin split (27K–50K–20K)-S-1 (c) were run as controls. The NH_2-terminal structure of the single 31K species is presented. (S) is residue tentatively assigned as serine.

using gradient gel electrophoresis (Figure 6A, lane a). The pattern obtained clearly showed the progressive formation of a protein doublet of M_r 33K–31K, suggesting that trypsin cuts multiple vicinal bonds at the 63K–31K junction. Both peptide bands were originated from the COOH-terminal part of the heavy chain as, like the skeletal 20K peptide (Suzuki & Morita, 1987), they could be overlayed with IAEDANS-labeled F-actin after electrophoretic transfer onto nitrocellulose. As shown in Figure 6A, lanes T2 and t2, the fluorescent actin stained also the thrombin-generated COOH-terminal 30K fragment of skeletal S-1 used as control (Chaussepied et al., 1986a), but it did not react at all with the NH_2-terminal 63K or 68K heavy chain counterparts.

The fractionation of the tryptic digest by a first reverse-phase HPLC led to a fraction containing the two peptide materials (Figure 6B, lane a). Following a second HPLC step, the peptide with the faster mobility could be isolated in a reasonably pure state (Figure 6B, lane d) and was submitted to amino acid microsequencing. The NH_2-terminal 17-residue sequence of this peptide entity is presented in Figure 6B. This sequence seems to be unique to the scallop S-1 heavy chain molecule, as no homologous stretches could be observed in the reported head sequences of other striated muscle myosins (Karn et al., 1983; Strehter et al., 1986; Maita et al., 1987). The apparent binding of actin to the nitrocellulose-immobilized COOH-terminal 31K and 24K tryptic fragments of the scallop S-1 encouraged us to isolate the 24K peptide light chains complex in order to probe quantitatively and more specifically its eventual interaction in solution with F-actin, in comparison with the homologous skeletal complex previously described

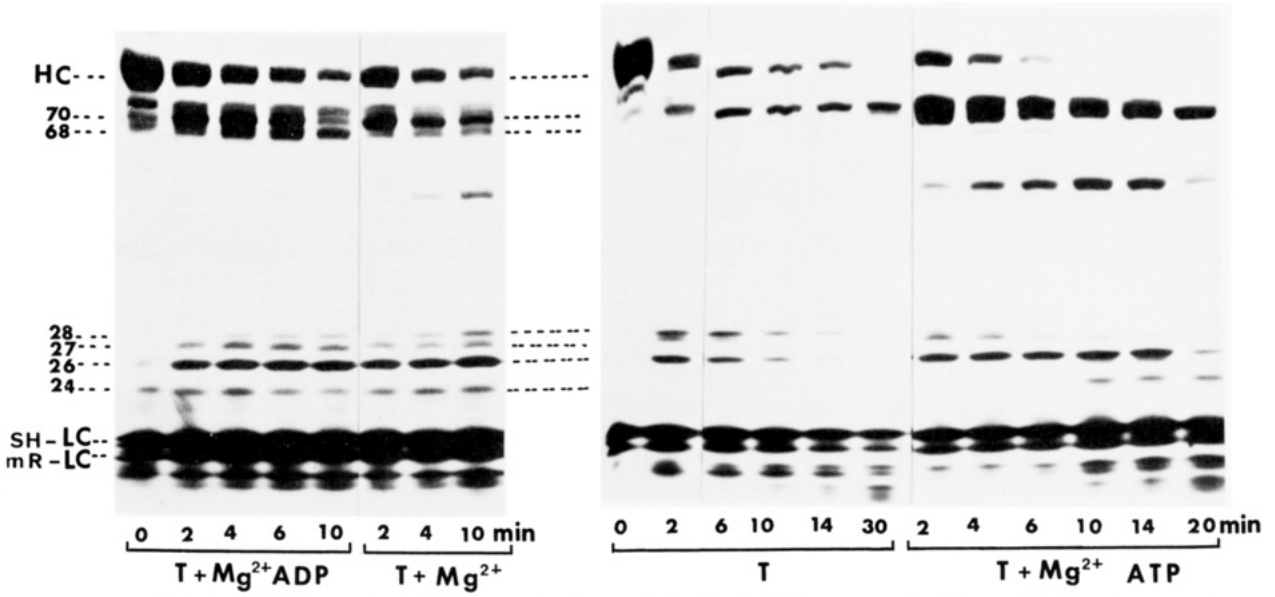

FIGURE 7: Nucleotide-induced fragmentation of the scallop S-1 heavy chain. S-1 (+LC) was treated with trypsin in the absence and presence of Mg^{2+}, Mg^{2+}-ADP, or Mg^{2+}-ATP (5 mM) under the conditions described in Figure 6A. At the times indicated, the digests were analyzed by gradient gel electrophoresis. T is native S-1.

(Chaussepied et al., 1986d). Using the general procedure reported by these authors, we did obtain reproducibly pure, soluble 24K fragment light chains preparations. However, using cosedimentation, turbidity measurements, and EDC cross-linking, under conditions identical with those employed for the skeletal 20K peptide (Chaussepied et al., 1986d), we failed to observe any binding of F-actin to the scallop 24K peptide preparations. These results suggest that the soluble scallop 24K region seems to lose its potential recognition ability for actin upon dissociation from the whole S-1 heavy chain.

Influence of Nucleotides on the Tryptic Cleavage of the Scallop S-1 Heavy Chain. The relationship between the ATPase inhibition and the actin-induced selective scission of the scallop S-1 heavy chain at the 65K–31K junction (Szentkiralyi, 1984) together with the known influence of nucleotides on the tryptic sensitivity of the skeletal S-1 heavy chain close to the 50K–20K junction (Labbé et al., 1984; Applegate & Reisler, 1984; Mocz et al., 1984; Mornet et al., 1985) led us to study the impact of nucleotide binding on the tryptic cleavage of the scallop S-1. The fragmentation patterns analyzed by gradient gel electrophoresis are presented in Figure 7. The addition of Mg^{2+}-ATP and Mg^{2+}-ADP to S-1 increased significantly the rate of the heavy chain degradation and, most probably, it changed also the nature of the proteolytic sites. Thus, the heavy chain was rapidly cut into 70K and 26K species; the latter product, present also in the control, would represent the COOH-terminal fragment that migrates as a 24K entity in nongradient gels (Szentkiralyi, 1984). The 70K material was further converted into a new peptide with a mass about 68K together with the production of a smaller peptide with M_r of 48K that was readily observable in the digests containing ATP. Densitometric measurements showed, indeed, that ATP has the most prominent effect on the rate of the breakdown of the heavy chain and the concomitant formation of the 70K and 68K bands. Furthermore, the presence of Mg^{2+} and Mg^{2+} nucleotides increased the stability of the 26K species, which was overdigested after a few minutes in the control, in agreement with the previous observations describing the tryptic degradation of the COOH-terminal 24K fragment into 14K–12K pieces (Szintkiralyi, 1984; Bennett et al., 1984). Nevertheless, the intensity of the 26K band became negligible in the digests conducted for 20–30 min in the presence of Mg^{2+}-ATP, whereas the 68K species remained relatively stable. These particular digests, which lack an intact 26K–24K fragment, were exhaustively dialyzed in the cold until free of nucleotide. The protein material was mixed with 2–4-fold molar excess of F-actin and then centrifuged in a Beckman airfuge. Gel electrophoretic analyses showed all the original digest to be present in the supernatant, not at all combined with actin. We conclude that in the absence of an intact COOH-terminal 24K domain the nucleotide-induced 68K species, which must correspond to the NH_2-terminal counterpart of the scallop S-1 heavy chain, is unable to interact significantly with F-actin.

Discussion

In the present study the cross-linking of a soluble native myosin to F-actin was achieved for the first time, using the EDC approach. EDC-mediated cross-linking of actomyosin in skeletal muscle fibers has also been recently used for investigations on the fiber stiffness (Tawada & Kimura, 1986). The production of a covalent complex between actin and the Ca^{2+}-sensitive molluscan myosin offers the advantage that it may assist in defining further the mechanisms linked with the self-regulation of this myosin. When this work was under way, EDC-catalyzed cross-linking between rabbit skeletal actin and scallop HMM or S-1 was briefly reported (Jackson et al., 1986). For both myosin and HMM, cross-linking to actin caused an elevated activation of the Mg^{2+}-ATPase. However the reported cross-linking of HMM led to a concomitant loss in Ca^{2+} sensitivity, whereas for the myosin the Ca^{2+} sensitivity was not suppressed. The milder experimental conditions we employed are the most likely contributory factor for this difference, but the proteolytically produced HMM could be also more sensitive to the EDC reagent than the intact parent myosin. The EDC treatment of myosin alone did not alter the Mg^{2+}-ATPase, although we did not test its influence on the intrinsic Ca^{2+} sensitivity (Wells & Bagshaw, 1985). The initial Mg^{2p}-ATPase stimulation, observed in the absence of Ca^{2+} for the covalent actomyosin, could be explained by assuming a conformational change of the light chains toward an on state under the influence of the permanently bound actin. In this regard, it is noteworthy that photochemical cross-linking between scallop myosin light chains was shown to result also

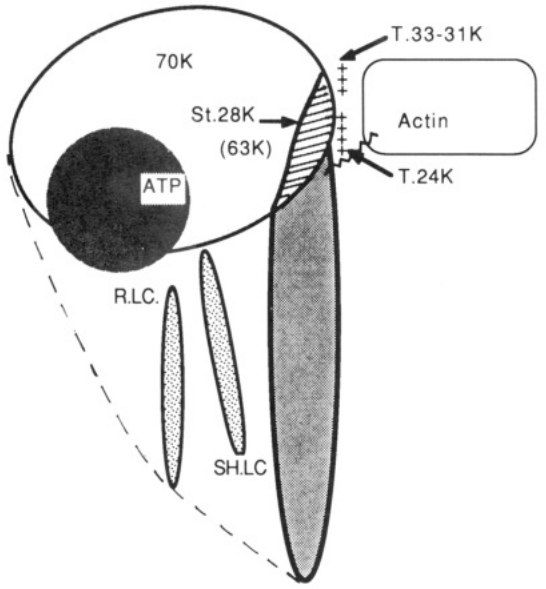

FIGURE 8: Diagram representing the positioning of actin and ATP relative to positively charged sites (++) on the surface of the 24K–33K heavy chain segment of scallop S-1. These sites are highly susceptible to trypsin (T) with a conformation sensitive to the binding of actin or nucleotide. The integrity of this conformation is essential for actin activation of S-1 and for ATP hydrolysis. (〰) indicates EDC cross-linking sites; (St) is the cleavage site of staphylococcal protease. For details, see text.

in a reduction of the Ca^{2+} sensitivity of the ATPase activity (Hardwicke et al., 1983). However, in the course of the EDC reaction with myosin or S-1, no products corresponding to inter-light-chain cross-linking were observed. The Ca^{2+}-induced increase of the ATPase activity could reflect either of the two following processes. First, Ca^{2+} binding to the cross-linked myosin heads has induced a further rearrangement of the regulatory light chains. Alternatively, if only one head of myosin has been cross-linked to actin with a complete loss of Ca^{2+} sensitivity, the second non-cross-linked head would remain tethered and fully regulated; thus, its ATPase could be activated, in the presence of Ca^{2+}, by the high local concentration of the adjacent actin filament (Hackney & Clark, 1984). Papain digestion of the isolated covalent actomyosin complex would permit us to distinguish whether cross-linking involves one or two myosin heads. In any case, the cross-linked actomyosin preparation appears useful for studies on the Ca^{2+} regulation of the scallop myosin ATPase in the presence of an infinite actin concentration.

Actin became cross-linked only to the S-1 heavy chain and not at all to the light chains, in agreement with the observations of Jackson et al. (1986). The covalent actin–heavy chain adduct migrated on electrophoretic gels as a triplet band, in contrast to the doublet observed for the skeletal S-1, which corresponds to actin cross-linked on either the 50K or 20K heavy chain fragment. Since the COOH-terminal 24K heavy chain segment is the main part of scallop S-1 involved in cross-linking, this heterogeneity may result from cross-linking at multiple sites on actin and/or the heavy chain. The cross-linking of the latter fragment matches well with the protection afforded by actin against the tryptic cleavage of the 70K–24K junction and the accompanying loss of the actin-dependent ATPase (Szentkiralyi, 1984). However, the covalent binding of actin is not followed by Mg^{2+}-ATPase stimulation when the scallop heavy chain is split by trypsin at the 63K–31K site, in agreement with the reported loss of intrinsic K^+- and Ca^{2+}-ATPases resulting from this cleavage (Szentkiralyi, 1987). Since ATP readily dissociates actin complexed to the (63K–31K)-S-1, the activity loss is likely to be due not to a deficient nucleotide binding but rather to the alteration of other kinetic steps of the ATPase reaction. The similar inactivation of the skeletal thrombic (68K–30K)-S-1 was found associated with the suppression of ATP hydrolysis (Chaussepied et al., 1986b). The primary structure of the scallop myosin heavy chain is as yet unknown. The NH_2-terminal sequence we have determined for the 31K fragment will permit the future localization of the inhibitory clip site within the covalent structure of the S-1 heavy chain. The data we obtained with the *S. aureus* protease digestion suggest that the functioning of the scallop ATPase site is more closely connected with the region surrounding this site than with other parts of the 31K–24K difference segment. The NH_2 terminus of 31K peptide is obviously within the central 50K heavy chain region about 7K apart from its C terminus. The known sequences of myosin S-1s from rabbit, chicken, rat, and nematode striated muscles do not exhibit apparent homologies in that part of the heavy chain relative to the determined 31K peptide sequence. On the other hand, the actin-dependent tryptic cleavage at the 63K–31K site is a feature observed only in the scallop S-1. Because the ATPase site of this enzyme is uniquely modulated by calcium, it could be that the particular structure of the 63K–31K region has been imposed by the regulation properties of the molluscan myosin. The overall data for the interaction and chemical cross-linking between actin and the scallop S-1 heavy chain are summarized in Figure 8.

Finally, the failure of the soluble, isolated 24K fragment–light chain complex to combine with F-actin is another peculiarity of the scallop S-1 heavy chain. It is unlikely that this behavior results from some detrimental effects of the isolation procedure on the peptide structure. Our data are rather consistent with the recently reported observation that actin does not bind to the nub fragment of scallop myosin which includes at the NH_2 terminus the 24K region and which was produced proteolytically without the use of a denaturant (Castellani et al., 1987). Furthermore, overdigested scallop S-1 populations, containing an intact 24K fragment associated with a fragmented N-terminal heavy chain counterpart, were not able to cosediment with actin (Bennett et al., 1984). The overall findings imply that in scallop S-1 internal interactions between the 24K region and other crucial parts of the heavy chain are essential for the firm and specific binding of actin. This idea is further supported by the failure of our attempts to combine actin with the 24K peptide deficient S-1 species formed upon tryptic digestion of the S-1–Mg^{2+}-ATP complex. The latter result indicates that actin cannot bind to the NH_2-terminal 68K heavy chain segment alone, and any interaction between actin and this region in the intact S-1 would require the structural integrity of the COOH-terminal 24K heavy chain segment.

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Registry No. ATPase, 9000-83-3; ATP, 56-65-5; Mg-ATP, 1476-84-2; Mg-ADP, 7384-99-8; Ca, 7440-70-2; Mg, 7439-95-4.

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