New Subfragment 1 of Skeletal Muscle Myosin Obtained by Thrombin Cleavage†

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ABSTRACT: The head of the myosin molecule (i.e., subfragment 1 with a heavy chain of 95 kDa) is usually obtained by chymotryptic cleavage in the presence of a divalent cation chelator. In the present work, we used another specific proteolytic enzyme, thrombin, to produce a limited cut within the myosin molecule, resulting in a new species of N-terminal fragment. Treatment of skeletal muscle myosin yielded a 97-kDa split heavy chain associated with intact light chains, corresponding to a single cut. The ATPase activities of this new S-1 derivative were slightly affected by the breakdown. It recognized actin in an ATP-dependent manner, as expected, with an affinity 2–5 times higher than that of the usual chymotryptic S-1 preparation but with a very different electron microscopic pattern. Functional differences are noted, and we involve them more precisely in relation to possible structural aspects of the additional C-terminal segment extending the usual S-1 heavy chain from 95 to 97 kDa.

Demonstration of the direct involvement of the myosin head in actin and nucleotide recognition has been useful in determining the main portion involved in the chemical processes of this particularly elongated and asymmetric molecule (Botts et al., 1989).

Following the pioneering work of Balint et al. (1975) based on enzymatic cleavages of myosin, we and others have extended the method, obtaining different specific cutting patterns of the subfragment 1 heavy chain (Mornet et al., 1979, 1984; Yamamoto & Sekine, 1979a; Applegate & Reisler, 1983).

Using limited proteolysis, it was possible to study the properties of each fragment obtained in relation to its ability to bind actin and/or nucleotide (Mornet et al., 1981a; Yamamoto & Sekine, 1979b; Chaussepied et al., 1986). From these studies, the architecture of the myosin head was found to involve a three-domain structure (Yanagisawa et al., 1987; Audemard et al., 1988).

During the same period and more recently, other workers have carefully investigated the properties of myosin subfragment 2 (Rodgers & Harrington, 1987) as well as the behavior of heavy meromyosin, which contains the intact S-1–S-2[1] junction along with the phosphorylated light chain (LC_2). This light chain could play an important role in the processes regulating ATPase activity in skeletal muscle myosin (Cardinaud, 1985) and also in smooth muscle myosin (Sellers et al., 1985; Citi et al., 1987), but the myosin head does not retain its anchorage site (Mocz et al., 1982) when it is isolated as a chymotryptic S1.

Thus, progressive enzymatic degradation of myosin is of great interest since it allows a determination of the properties remaining in shorter and shorter segments. This has been very well illustrated recently by the gallery of microscopic images presented by Kuczmarski et al. (1988). On the other hand, studying longer heads could also provide important information. The objective of the present investigation was to obtain a longer C-terminal portion of heavy chain as an S-1 molecule and to determine the resulting properties (in comparison with the usual chymotryptic S-1 heavy chain), particularly with respect to the role of the LC_2 light chain.

Materials and Methods

Protein Preparations. Myosin was isolated from rabbit back and hind leg muscles according to the method of Offer et al. (1973).

S-1 was obtained by digestion of myosin filaments with Worthington α-chymotrypsin as described by Weeds and Taylor (1975). It was purified on Sephacryl S-200, and elution was carried out in 50 mM Tris-HCl buffer (pH 7.5) containing 0.1 mM NaN_3.

Digestion of myosin (4 mg/mL) with Tebu human thrombin was carried out at a protease/myosin weight ratio of 1:100 in 25 mM Tris-HCl and 250 mM KCl, pH 8.0, in the presence of 5 mM EDTA for 4 h. The digest was dialyzed overnight against 25 mM Tris-HCl, pH 7.0. After centrifugation at 200000*g* for 2 h at 4 °C, the supernatant was purified on Sephacryl S-200 and eluted with 25 mM Tris-HCl and 0.1 mM NaN_3, pH 7.0. However, we noted that the yield of the digestion was 10–30 mg/g of myosin depending on the thrombin sample used.

Myosin and S-1 were labeled with *N*-(iodoacetyl)-*N'*-(5-sulfo-1-naphthyl)ethylenediamine (1,5-IAEDANS) as reported by Mornet et al. (1981b).

Arginase C submaxillaris proteolysis of chymotryptic and thrombic S-1s was obtained as described by Bertrand et al. (1989), except that S-1 was digested with arginase C at a protease/substrate weight ratio of 1:180 overnight at 4 °C.

F-Actin was prepared as in Eisenberg and Kielley (1974) and was labeled with 1,5-IAEDANS as described by Takashi et al. (1976).

Protein concentrations were determined by using $A^{1\%}_{280nm}$ = 7.5 (Wagner & Weeds, 1977) for chymotryptic and thrombic S-1 and $A^{1\%}_{280nm}$ = 11.0 for F-actin.

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[1] Abbreviations: IAEDANS, *N*-(iodocetyl)-*N'*-(5-sulfo-1-naphthyl)-ethylenediamine; MES, 2-(*N*-morpholino)ethanesulfonic acid; DTE, dithioerythritol; EDC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide; PDG, phenyldiglyoxal; S-1, myosin subfragment 1; EDTA, ethylenediaminetetraacetic acid; $NaDodSO_4$, sodium dodecyl sulfate; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; ATPase, adenosine-5'-triphosphatase.

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Polyacrylamide Gel Electrophoresis. $NaDodSO_4$–polyacrylamide gradient gel electrophoresis (5–18%) was carried out as described by Mornet et al. (1981a). The gels stained by Coomassie blue were scanned with a Shimatzu Model CS 930 high-resolution gel scanner equipped with a computerized integrator.

ATPase Activities. The ATPase activities of S-1 were measured as described by Mornet et al. (1979).

Cross-Linking Reactions. F-Actin (2 mg/mL) and S-1 (2 mg/mL) were mixed in 0.1 M MES in the presence of 10 mM 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDC) as previously described (Mornet et al., 1981b).

F-Actin and S-1 were cross-linked by using phenyldiglyoxal as described by Bonet et al. (1988a).

Determination of the Actin Affinity Constant. The affinity of actin for the thrombic and the chymotryptic S-1 preparations was measured by sedimentation in a Beckman airfuge essentially as described by Bonet et al. (1988b). Native S-1s (1 μM) were mixed with varying concentrations (0–3 μM) of F-actin in 25 mM Tris buffer, pH 7.0, 10 mM KCl, 2.5 mM $MgCl_2$, and 0.5 mM DTE. After sedimentation at 178000*g* for 35 min at 22 °C, the fraction of S-1 remaining in the supernatant was determined by densitometric measurement of the 95- or 97-kDa heavy chain band present on $NaDodSO_4$ electrophoretic gels.

Association and dissociation reactions of the complexes of actin with native chymotryptic or thrombic S-1s were also studied by turbidimetry at 400 nm, 20 °C, in the absence or presence of 5 mM $MgCl_2$ plus 1.5 mM ATP as described by White and Taylor (1976). The values are arbitrary units.

C-Terminal Analyses. For C-terminal analyses, digestion with carboxypeptidases A and B was carried out according to the method of Morgan and Henschen (1969).

Electron Microscopy. Complexes of F-actin (0.1 mg/mL) with chymotryptic S-1 or thrombic S-1 were mixed in 50 mM Tris-HCl, 50 mM KCl, 0.1 mM $CaCl_2$, and 0.1 mM NaN_3, pH 7.5, in a weight ratio of 1:2 for 15 min at room temperature and then negatively stained with 1% aqueous uranyl acetate on Formvar/carbon-coated 300-mesh grids as previously described (Mornet et al., 1988). To show the reversibility of the phenomenon, an ATP/$MgCl_2$ solution was added to the former solution at final concentrations of 5 mM ATP (adjusted to pH 7.5) and 1 mM $MgCl_2$. Actin, present at 0.05 mg/mL, was incubated for 5 min at room temperature. All observations were made by using a JEOL 2000 EX electron microscope at 80 kV with an objective aperture of 20 μm.

Titration of Tryptophan Residues. Comparative spectrophotometric determinations of the tryptophan contents of chymotryptic and thrombic S-1 were carried out according to the method of Edelhoch (1967), and the reported value corresponds to an average of eight determinations.

Results

(1) Cleavage of Myosin by Thrombin. Skeletal muscle myosin heavy chain treated with thrombin in the presence of EDTA was partially cleaved into 130- and 97-kDa fragments. The light chains were unaffected by thrombin cleavage when associated with the heavy chain. After fluorescent labeling of native myosin, we verified that the 1,5-IAEDANS (1.9 mol/mol of myosin) was incorporated into the 97-kDa thrombic segment (corresponding to the SH_1 modification in the fragment of M_r = 20000 known as the C-terminal part of the usual chymotryptic S-1 heavy chain, as previously reported; Mornet et al., 1985; Audemard et al., 1988).

After 4 h, proteolysis was achieved, and further addition of thrombin to the medium did not increase the yield of the cut. The presence of 5 mM $MgCl_2$ completely inhibited proteolysis. Although some traces of 140-kDa material were present in our myosin preparation, the new S-1 thrombin did not arise from this source, as shown in the time course of Figure 1.

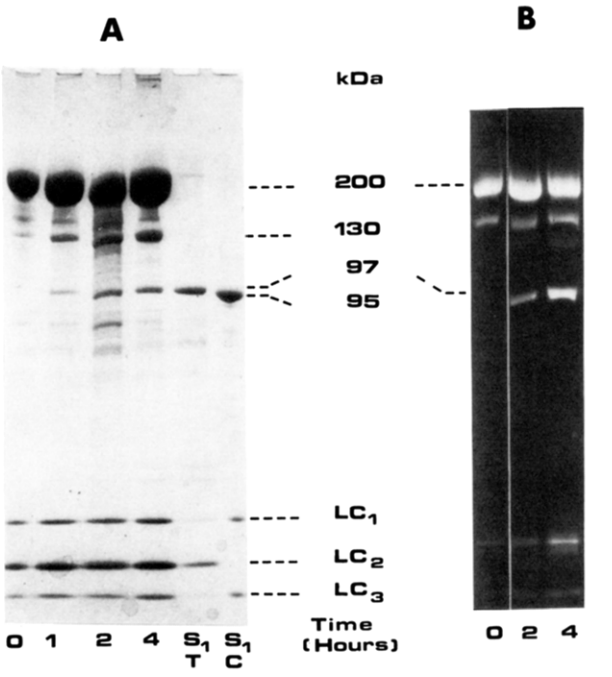

FIGURE 1: Time course of skeletal myosin proteolysis by thrombin. At the indicated times during cleavage, samples of skeletal myosin were analyzed by polyacrylamide gel electrophoresis. (A) Gel stained by Coomassie blue showing a single proteolytic cleavage within the 200-kDa heavy chain, resulting in two fragments of 130 and 97 kDa. The time course of the cleavage is shown. (B) Same gel viewed under ultraviolet light. The myosin heavy chain labeled at its SH_1-reactive thiol by 1,5-IAEDANS is degraded into 97-kDa fluorescent products and an unlabeled 130-kDa fragment. Comparative migration on the same gel for the chymotryptic S-1 (S_1C) and the unpurified thrombic S-1 (S_1T).

The 97-kDa region containing the fluorescent label was identified as the N-terminal portion of the molecule. As can be seen in Figure 1, after the split mixture was dialyzed in a low ionic strength buffer and subjected to centrifugation, a soluble mixture of 97-kDa fragment with intact myosin light chains was found in the supernatant, with heavy chain longer than that of chymotryptic S-1.

Thrombin, a specific proteolytic enzyme, produced a single and partial proteolytic cleavage in the skeletal muscle myosin heavy chain. This result is important, since under the same conditions gizzard smooth muscle myosin heavy chain was completely resistant to thrombin attack. This shows the importance of the amino acid sequence for the efficacy of this protease and demonstrates once again (Chaussepied et al., 1986; Henry et al., 1985) that although all myosin molecules contain the two "connector regions" between the domain-like segments of their head portion, these highly variable regions are not at all affected by thrombin (Audemard et al., 1988; Bonet et al., 1987).

The specificity of the thrombin cleavage in skeletal muscle myosin could correspond to a partial heterogeneity of the 200-kDa heavy chain as previously revealed by the presence of myosin isozymes (d'Albis et al., 1986; Hofmann et al., 1988) and by the incomplete *Staphylococcus aureus* V_8 proteolysis (only 90%) reported by Chaussepied et al. (1984).

Although the resulting protein mixture was found to contain a 97-kDa fragment with all myosin light chains intact, we subjected the preparation to purification by chromatography

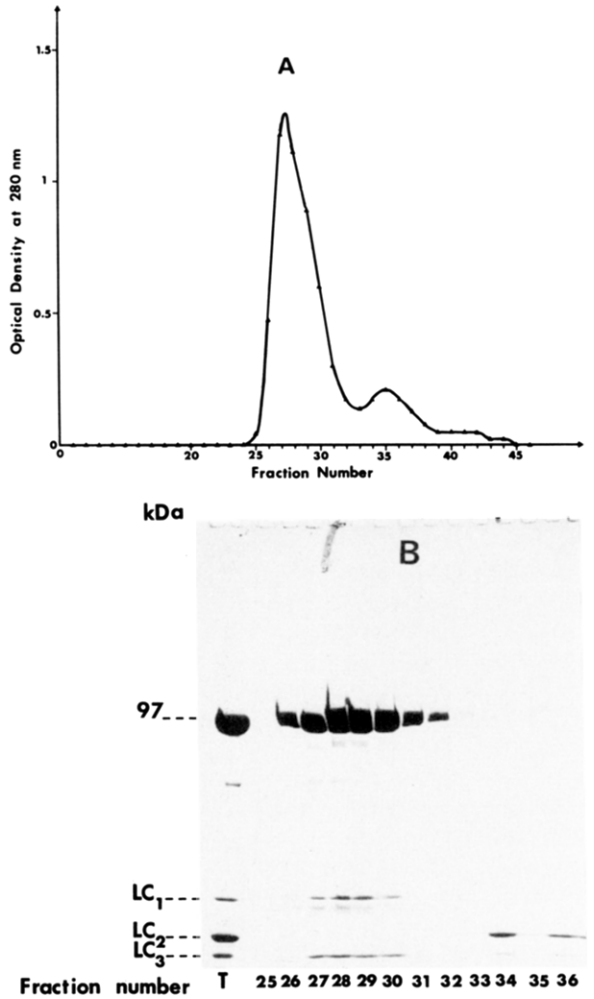

FIGURE 2: S-200 chromatographic separation of the soluble head portion from thrombin-cleaved myosin. Separation of thrombin-cleaved myosin by chromatography on a Sephadex S-200 column was monitored at 280 nm for thrombic S-1 purification. (A) Elution pattern: each fraction corresponds to 2 mL. Purified thrombic S-1 corresponds to fractions 26–31. (B) Indicated fractions as analyzed by electrophoresis on polyacrylamide slab gel under denaturing conditions. The regulatory light chain (LC_2) was adequately separated by this procedure, showing that it results only from EDTA extraction (this chelating agent is required during myosin cleavage by thrombin).

on a Sephacryl S-200 column as described under Materials and Methods. This step was undertaken mainly because it is known that an LC_2 preparation can be obtained from myosin by EDTA treatment followed by centrifugation (Pastra-Landis & Lowey, 1986).

As shown in Figure 2A, S-200 chromatography gave an elution pattern with a shoulder around fractions 33–36, and the gel pattern (Figure 2B) indicates that the N-terminal soluble part of myosin after thrombin cleavage consisted of a 97-kDa fragment associated with alkaline light chains, whereas LC_2 was only obtained from EDTA extraction and corresponded to fractions 26–31. This entity was then analyzed, and carboxypeptidase B treatment showed an arginine residue at the carboxyl-terminal end (-Arg).

The same C-terminal determination of the usual chymotryptic S-1 (where the heavy chain corresponds to a 95-kDa fragment) revealed its COOH end to be (-Tyr-Phe). The thrombic S-1 possessed 6.5 ± 0.1 tryptophan residues before S-200 chromatography but 5.9 ± 0.1 after this purification. Spectral determination gave a value of 5.8 ± 0.1 for chymotryptic S-1 tryptophan content. Then by this step we have eliminated from our S-1 preparations all native or digested

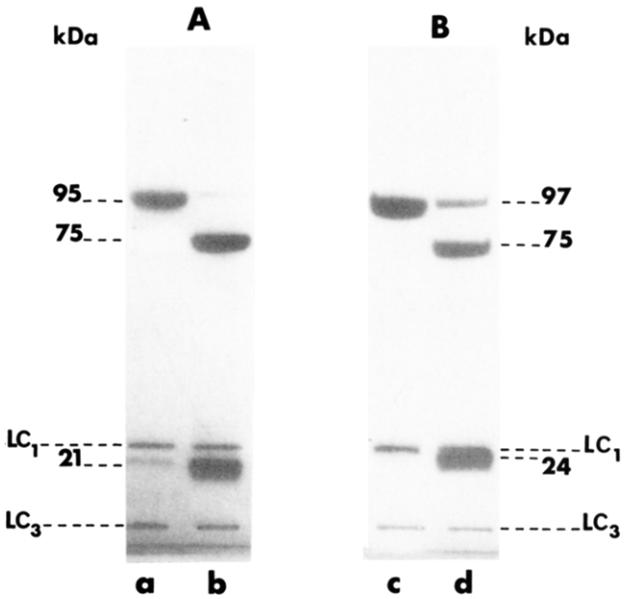

FIGURE 3: Time course of submaxillaris arginase C cleavage of chymotryptic S-1 compared to thrombic S-1. The pattern of specific submaxillaris arginase C proteolysis of chymotryptic S-1 heavy chain (A) revealed a C-terminal portion migrating as a 21 000 product, while thrombic S-1 heavy chain (B) gave rise to a new 24 000 C-terminal product.

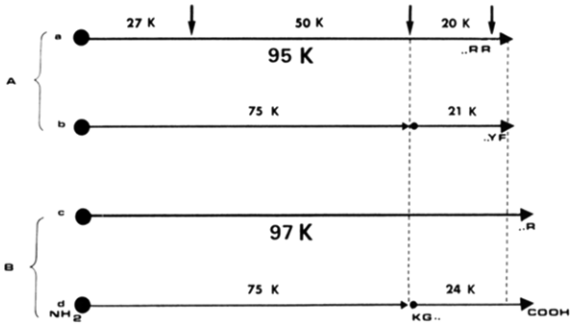

FIGURE 4: Schematic representation of the proteolytic event occurring on chymotryptic (A) or thrombic (B) S-1 heavy chains. Arrows correspond to the trypsin cutting sites on the myosin head heavy chain [see the review Audemard et al. (1988)]. (b) and (d) correspond to the arginase C proteolytic sites on chymotryptic and thrombic S-1, respectively. The vertical dashed line shows that thrombic S-1 is elongated at its C-terminal end compared to chymotryptic S-1. Large funnels correspond to the carboxypeptidase determination of the C-terminal extremities (a, c) or N-terminal sequencing of the corresponding fragments.

LC_2 light chain. The difference in the molecular masses (97 and 95 kDa) of these two S-1 heavy chains and in their C-terminal ends was confirmed by subjecting the two preparations to additional mild proteolysis with submaxillaris arginase C protease. With the chymotryptic S-1 heavy chain of 95 kDa the proteases showed a single proteolytic cleavage at Lys 640–Lys 641, as reported recently (Bertrand et al., 1989). This cleavage produced two fragments, the N-terminal 75-kDa and the C-terminal 21-kDa fragments (Figure 3A, lane b). The new thrombic S-1, with its 97-kDa heavy chain segment, was cleaved into a similar 75-kDa N-terminal fragment, but its C-terminal part migrated as a new 24-kDa fragment (Figure 3B, lane d). This result demonstrates that the thrombic S-1 is longer than chymotryptic S-1 by about 20–30 residues in its C-terminal end. We also noted here that the 21-kDa fragment must contain the same C-terminal end as the intact chymotryptic 95-kDa heavy chain, which is not the case of the C-terminal 20-kDa fragment obtained by trypsin cleavage. If this were not true, the new 97-kDa heavy chain would not show

Table I: Comparison of Ca^{2+}-, K^+-, and Mg^{2+}-Dependent ATPase Activities of the Chymotryptic and Thrombic S-1s Expressed in μM P_i mg^{-1} min^{-1}

S-1	ATPase activities				activation factor
	Ca^{2+}	K^+	acto Mg^{2+}	Mg^{2+}	
chymotryptic	1.13	5.58	1.88	0.049	×50
thrombic	1.07	6.37	1.82	0.03	×60

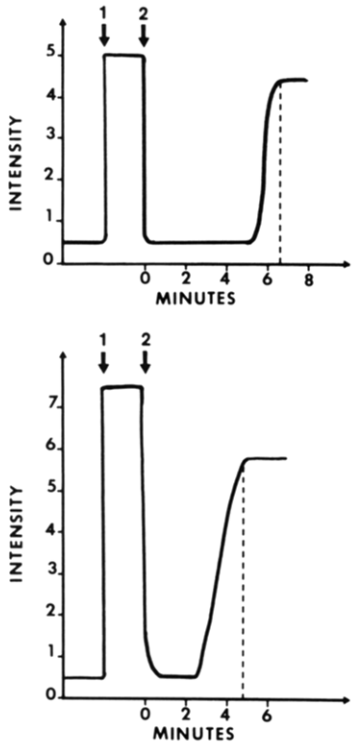

FIGURE 5: Comparative light scattering of the complex of actin with chymotryptic or thrombic S-1. Measurement at 400 nm, 20 °C, in the absence or presence of 5 mM $MgCl_2$ plus 1.5 mM ATP on a Turner fluorescent apparatus. The values are arbitrary. S-1 (0.1 mg) in 10 mM Tris-HCl, 100 mM KCl, and 1 mM $MgCl_2$, pH 8.0 (2 mL), were mixed with 0.2 mg of F-actin. At arrow 1, F-actin was added to (A) chymotryptic S-1 or (B) thrombic S-1. At arrow 2, ATP-Mg was added to either acto–S-1 complex, and dissociation was monitored until the reassociation step.

any splitting differences from the 95-kDa fragment when subjected under the same conditions to submaxillaris arginase C protease (see diagram in Figure 4).

(*2*) *Comparison of the ATPase Activities and Actin Binding Properties of Native Thrombic S-1 and Native Chymotryptic S-1 Preparations.* Considering the small difference in the sizes of the thrombic and chymotryptic S-1 heavy chains, we did not expect dramatic differences in the ATPase activities of the two S-1 preparations. As shown in Table I, the basal ATPase activities were indeed rather similar, although the magnesium-dependent actin activation of the acto–S-1 complex was stronger with thrombic S-1 than with chymotryptic S-1.

Using turbidity measurements at 400 nm, we then examined association and dissociation by ATP-Mg^{2+} of the complexes of native chymotryptic S-1 or thrombic S-1 with actin. As shown in Figure 5, both mixtures were dissociated by ATP-Mg^{2+}, but thrombic S-1 showed higher turbidity and, surprisingly, it reassociated more rapidly with actin.

We then investigated the actin binding properties of this new S-1 preparation using airfuge centrifugation with increasing actin concentrations. Average of densitometric measurements of the respective fragments in the supernatant indicated a small but significant difference (2.5-fold) in their actin affinity: $K_a = (4 \pm 0.9) \times 10^6\ M^{-1}$ instead of $K_a = (1.6 \pm 0.9) \times 10^6\ M^{-1}$.

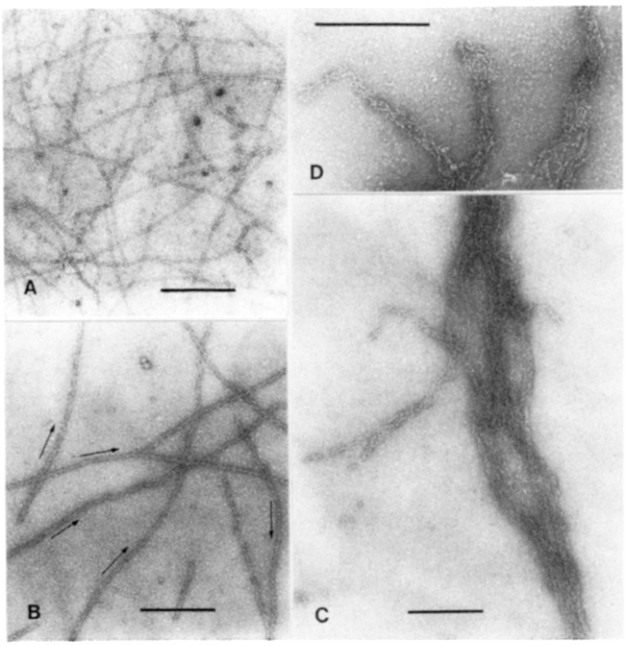

FIGURE 6: F-Actin decoration with chymotryptic S-1 or with thrombic S-1. (A) Electron micrograph of rabbit skeletal F-actin filaments. (B) Electron micrograph of the acto–chymotryptic S-1 complex. Decorated actin filaments reveal clearly oriented arrow heads (black arrows). (C, D) Electron micrographs of the acto–thrombic S-1 complex. (C) Actin filaments are fully decorated but always found in assembled structures. (D) High magnification reveals that always several decorated actin filaments are entangled. Magnifications of all micrographs are identical with the exception of that for panel D, shown at high magnification. Bars represent 0.5 μm.

Covalent association of actin with the 97-kDa thrombic heavy chain was obtained by using either the carbodiimide activation procedure or the PDG chemical cross-linker. The product (97-kDa fragment-actin) was found to migrate as a doublet (170–190 kDa) in the same manner as the actin product obtained with 95-kDa chymotryptic heavy chain by using EDC (Mornet et al., 1981) and as a single band of about 210 kDa by using PDG (Bonet et al., 1988a; results not shown).

Chymotryptic S-1 forms complexes with actin, and microscopic observation of the association revealed the regular arrowhead decoration, as shown in Figures 6B and 7A,B. No free F actin was found, and the pattern of the filaments is similar when free (Figure 6A) or fully decorated (Figures 6B and 7B). This decoration was always found identical with different actin/S-1 weight ratio (1:2, 1:4). However, thrombic S-1, used the same day under the same conditions, produced only an aggregate structure (Figure 6C) resembling an entanglement of actin filaments rather than a bundling phenomenon (Figures 6C and 7C). No free decorated filaments were observed as shown in Figure 7C,D. The structures grew with incubation, before negative staining. All the actin filaments were decorated, but no arrowheads were clearly oriented as pointed out in Figure 7D (black triangle), on a rare single filament associated with the entangled structures; the decoration showed an irregular disoriented pattern. The two phenomena were completely reversed in the presence of 5 mM ATP-Mg (Figure 7E).

Conclusion

We show here that limited cleavage can be obtained with thrombin in the presence of EDTA, producing a new S-1 preparation from skeletal muscle myosin. When the resulting head portion was isolated by gel chromatography, it was found

Table II: Comparison of Skeletal Muscle Myosin (A) and Smooth Muscle Myosin (B) Sequences at the S-1–S-2 Junction[a]

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             C                                           T
(A)  MKLYFKIKPLLKSAETEKEMANMKEEFEKTKESLAKAEAK [R] KELEEKMVALMQEKNDLQLQVQAEADSL
(B)  WRLFTKVKPLLQVTRQEEEMQAKDEELQRTKERQQKAEAE [L] KELEQKHTQLCEEKNLLQEKLQATELY
                    P

(A)  ADAEE- [R] QDLIKTKIQLEAKIKEVTE [R] AEDEEEINAEL-AKK [R] KLEDECSELKK
(B)  AEAEEM [R] VRLAAKKQELEEILHEMEA [R] IEEEEERSQQLQAEK [K] KMQQQMLDLEE
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[a] P corresponds to the papain cleavage site of smooth muscle subfragment 1. C corresponds to the chymotyptic cleavage site of skeletal muscle subfragment 1. T corresponds to the thrombic cleavage site. In boxes are all the arginine residues (R), i.e., potential targets that were found by carboxy-terminal analysis to be in the C-terminal end of our thrombic S-1. (A) From Capony and Elzinga (1981). (B) from Onishi et al. (1986).

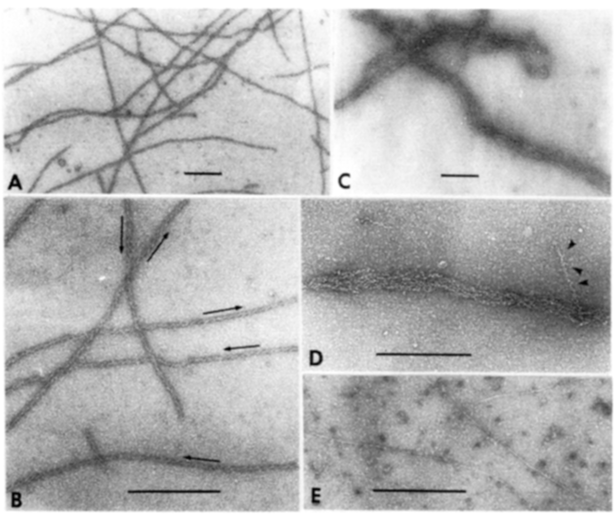

FIGURE 7: Electron micrographs of the acto–S-1 complexes. (A, B) Chymotryptic S-1 plus actin: decorated actin filaments were observed (A), which at higher magnification (B) showed clearly oriented arrowheads (black arrows). (C, D) Thrombic S-1 plus actin: a net of intermingled filaments (C) was formed consisting of fully decorated actin filaments with an irregular disoriented pattern [black triangle, shown in (D)]. Addition of ATP-Mg (E) to the thrombic S-1–actin complex caused a reversion of binding and the appearance of free isolated actin filaments. Identical reversion was also obtained with chymotryptic-S-1–actin complexes (data not shown). Bars represent 0.5 μm.

to have a longer heavy chain (97 kDa instead of 95 kDa for chymotryptic S-1 heavy chain) associated only with intact alkaline light chains. This small difference in the size of the thrombic S-1 heavy chain did not alter the basal activities of the molecule but slightly increased its magnesium-dependent actin-activated ATPase activity as well as its affinity for actin.

In spite of this elongated 2- or 3-kDa C-terminal portion, the thrombic S-1 was unable to retain the LC_2 light chain that binds the S-2 portion, elongating S-1 into the HMM molecule, as revealed by chemical cross-linking (Mocz et al., 1982). By use of immunoelectron microscopy, the LC_2 light chain has been further localized in the neck region of the myosin head (Winkelman et al., 1983; Winkelman & Lowey, 1986) where the scallop regulatory light chain is also located (Flicker et al., 1983).

Thus, this thrombic S-1 segment does not contain enough information for forming a complex with LC_2 light chain. The chromatography on the S-200 column resulted in a good purification of our thrombic S-1 from both native and degraded LC_2. However, we cannot exclude the possibility that this separation is due to the weakening of the HC–LC_2 interaction and that some information exists in our thrombic S-1 heavy chain for the LC_2 binding to occur but which is not strong enough for an efficient association. This poor binding could also be caused by the aggregation properties of the additional C-terminal portion of our thrombic S-1 heavy chain. We notice also that the tryptophan titration of thrombic and chymotryptic purified S-1 heavy chain gave us a discrepancy of one residue between the amount of five residues deduced from the heavy chain sequence (1–810) and the found values for the full-length heavy chains. This additional residue must be positioned between the RR C-terminal end of the 20-kDa heavy chain segment and the chymotryptic C-terminal heavy chain end, in agreement with the presence of two or three tryptophan residues found in many other myosin sequences (Mornet et al., 1989). It has strong actin binding properties as indicated by its higher affinity ($K_a = 4 \pm 0.9 \times 10^6$ M^{-1}) and more rapid reassociation into the acto–S-1 complex, as shown by turbidity measurements (Figure 5). This difference can be explained easily by the tendency of our thrombic S-1 to aggregate as shown in our microscopic images (Figures 6 and 7).

On the basis of the analysis of the S-2 sequence reported by Capony and Elzinga (1981), the additional C-terminal portion within the thrombic S-1 heavy chain probably corresponds to 20–30 residues, since there are only a few arginine residues in this region. Here we have taken advantage of the specificity of the thrombin cleavage. Skeletal muscle myosin was cleaved, and smooth muscle myosin was not. Thus, in Table II, which compares the two sequences spanning 20–30 amino acids beyond the S-1–S-2 junction, we have boxed in the only arginine residue corresponding to the thrombin target in the skeletal muscle myosin sequence, and we have found only one arginine residue absent in the smooth muscle myosin. Finally, the thrombin S-1 is assumed to be shorter than papain S-1, since the latter retains the LC_2 light chain while thrombin S-1 does not. However, we cannot precisely indicate the papain cleavage site because of the heterogeneity of the proteolysis. Nevertheless, the C-terminal papain end of smooth muscle myosin subfragment 1 (Onishi et al., 1986) shows its elongated portion, which is in agreement with our finding that mild proteolysis led to the isolation of a smooth muscle S-1 retaining its 20-kDa light chain (Bonet et al., 1987). This particular C-terminal end could contain part of the elastic properties of the S-1–S-2 portion reported by Ueno and Harrington (1985), and since thrombin cleavage was only possible when EDTA was present, the region must be structurally important for metal and LC_2 binding. Recent temperature-dependence

studies have demonstrated that the contribution of structural transitions in the flexible joint between the head and rod portions of the myosin molecule can be detected by using limited chymotryptic digestion (Pliska et al., 1989). Bundle formation of actin filaments was also detected by adding skeletal muscle myosin subfragment 1 (Ando & Scales, 1985; Ando, 1987), but our pictures of chymotryptic and thrombic S-1 obtained from the same actin preparation were completely different. The experimental conditions differed only with respect to ionic strength (50 mM KCl instead of 150 mM) and incubation time (15 min instead of 1–24 h). In our hands chymotryptic S-1 never produces the entangled structures that we observed with thrombic S-1. Intramolecular aggregation has in fact been observed on electron micrographs of rotary-shadowed LC_2-deficient myosin (Pastra-Landis & Lowey, 1986). These authors showed that the oligomeric structures correspond to two or more myosin molecules associated by the "neck" region of the head near the rod junction. Moreover, the relationship between the alkaline light chains and the LC_2 light chain has been shown to be important for the filament structure (Cardinaud, 1985), inducing a stabilizing effect on the myosin molecule (Pastra-Landis & Lowey, 1986) and preventing myosin aggregation. More recently, Chowrashi et al. (1989) have pointed out the direct LC_2 involvement in the assembly of skeletal myosin filaments as well as in the control of filament length described in skeletal and cardiac myosins (Margossian et al., 1987).

Thus, the new thrombic S-1 described here, elongated at the head–rod junction by only about 36 residues, appears to be able to entangle actin filaments by first making oligomeric structures of two or more S-1 molecules. This result sheds further light on the importance of the presence of the LC_2 on the myosin molecule at this junction.

We propose a possible role for the LC_2 light chains as a support for the S-1–S-2 regions, allowing the head portion to interact more efficiently with actin filaments, producing the regular arrowhead decoration of actin when complexed with HMM (Pastra-Landis & Lowey, 1986). In our case, skeletal muscle myosin heavy chain would appear to contain flexibility information in its S-1–S-2 portion (corresponding to 36 residues added to our thrombic S-1 heavy chain) that only LC_2 can control, since the presence of calcium makes the myosin heavy chain resistant to thrombin attack. This interpretation is in complete agreement with the observations of LC_2-deficient HMM reported by Pastra-Landis and Lowey (1986), which forms an oligomeric structure, indicating a clearly significant role for the LC_2 light chain within skeletal muscle myosin, particularly at the S-1–S-2 junction.

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Cryoenzymic Studies on Actomyosin ATPase. Evidence That the Degree of Saturation of Actin with Myosin Subfragment 1 Affects the Kinetics of the Binding of ATP†

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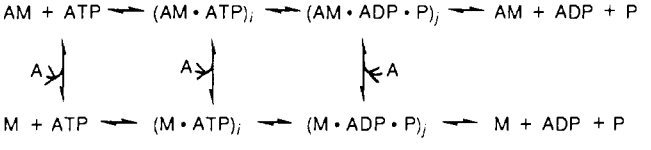
ABSTRACT: The initial steps of actomyosin subfragment 1 (acto-S1) ATPase (dissociation and binding of ATP) were studied at −15 °C with 40% ethylene glycol as antifreeze. The dissociation kinetics were followed by light scattering in a stopped-flow apparatus, and the binding of ATP was followed by the ATP chase method in a rapid-flow quench apparatus. The data from the chase experiments were fitted to E + ATP $\rightleftharpoons$ (K_1) E·ATP $\rightarrow$ (k_2) E*ATP, where E is acto-S1 or S1. The kinetics of the binding of ATP to acto-S1 were sensitive to the degree of saturation of the actin with S1. There was a sharp transition with actin nearly saturated with S1: when the S1 to actin ratio was low, the kinetics were fast ($K_1 > 300$ μM, $k_2 > 40$ s^{-1}); when it was high, they were slow ($K_1 = 14$ μM, $k_2 = 2$ s^{-1}). With S1 alone $K_1 = 12$ μM and $k_2 = 0.07$ s^{-1}. With acto heavy meromyosin (acto-HMM) the binding kinetics were the same as with saturated acto-S1, regardless of the HMM to actin ratio. The dissociation kinetics were independent of the S1 to actin ratio. Saturation kinetics were obtained with $K_d = 460$ μM and $k_d = 75$ s^{-1}. The data for the saturated acto-S1 could be fitted to a reaction scheme, but for lack of structural information the abrupt dependence of the ATP binding kinetics upon the S1 to actin ratio is difficult to explain. It is tentatively proposed that the transition is due to a cooperative phenomenon involving head–head interaction. It is suggested that in unsaturated acto-S1 the heads do not interact, even when attached to adjacent actin monomers, but that as further S1 binds and the saturation nears completion there is an abrupt structural change of the filament which is propagated along it. This is then transmitted to the heads which now interact. Therefore, it is proposed that when the heads interact, the ATP binding kinetics are slow (apparently this is always the case with acto-HMM) but when they do not, the kinetics are fast. The kinetic transition is not due to the presence of ethylene glycol: it was also obtained in an aqueous buffer with N^6-ethenoadenosine 5′-triphosphate. The results are discussed with reference to the structural information available in the literature.

Muscle contraction—as do the several processes involved in cell motility—depends on the cyclic interaction of actin and myosin, the energy for which is supplied by the hydrolysis of ATP[1] by the myosin heads. It is thought that movement is controlled by actin interacting in different ways with the different intermediates on the myosin ATPase reaction pathway. Therefore, to understand muscle contraction, one must obtain detailed information on the kinetics of the several processes involved in actomyosin ATPase.

Scheme I



It is very difficult to study directly the kinetic processes of organized systems. The use of caged compounds is an elegant way of attacking this problem [e.g., Goldman et al. (1984)

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[1] Abbreviations: ATP, adenosine 5′-triphosphate; ε-ATP, 1, N^6-ethenoadenosine 5′-triphosphate; P_i, inorganic orthophosphate; S1, myosin subfragment 1; HMM, heavy meromyosin; Tris, tris(hydroxymethyl)aminomethane; DTT, dithiothreitol.

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