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Structural and actin-binding properties of the trypsin-produced HMM and S1 from gizzard smooth muscle myosin

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The reaction of trypsin on the heavy chain of gizzard myosin and chymotryptic HMM was investigated under restricted fragmentation conditions. The three fragments of the head part with 29 kDa, 50 kDa and 26 kDa were isolated and identified. The 66 K heavy chain segment containing the S1–S2 junction was slowly but extensively degraded liberating a S1-like entity which lacked an intact COOH-terminal 26 kDa region; this isolated species displayed full intrinsic ATPase activities but little actin-binding ability. Tryptic HMM was also formed bearing a fragmented heavy chain and lacking the 20 kDa light chain. Its actin-activated ATPase was derepressed upon cleavage of the 66 kDa segment by papain. We propose that the integral 66 kDa heavy chain component is directly involved in the regulation of the gizzard actomyosin ATPase.

Smooth myosin *tryptic proteolysis* *S1–S2 junction* *Heavy chain fragment*

1. INTRODUCTION

During the course of our work on the chemical cross-linking between F-actin and the head heavy chain of gizzard myosin [1], we have been concerned with the preparation of tryptic myosin derivatives having defined structure and actin-binding properties. Earlier, the limited tryptic digestion of skeletal muscle S1 and HMM was shown to be a sensitive probe of the structure and conformation of the myosin head heavy chain [2–5]. On reacting trypsin with the smooth myosin, we have focused our attention on the mode of cleavage of the S1–S2 junction by this protease, because the neck region seems to be involved in the function of the smooth myosin. It presumably interacts with the 20 kDa light chain whose phosphorylation regulates kinetic steps of the actin-activated Mg^{2+}-ATPase of the head [6]; it also binds to the tail portion when the myosin adopts a folded conformation [7,8]. Previous proteolytic studies have shown the head-rod junction of the smooth heavy chain to be refractory to chymotrypsin [9,10], suggesting changes in the structure or conformation of this region as compared to its skeletal counterpart. Further information about the structure–function relationship within this area is, therefore, of special interest.

Herein, we show that trypsin splits the S1–S2 link at a slow rate but in an unusual manner; it occasion the extensive degradation of a 66 kDa heavy chain segment which encompasses the head and tail parts. The major structural and enzymatic features of the resulting isolated S1 and HMM species are presented.

2. MATERIALS AND METHODS

Myosin was prepared from fresh chicken gizzards by a combination of methods [11,12]. Chymotryptic HMM was isolated as in [10].

Gizzard S1 was prepared by digestion of pure myosin with mercuripapain (Sigma) and was purified by DEAE-cellulose chromatography as in [10]. Rabbit skeletal muscle F-actin was prepared as in [13].

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Digestion of gizzard myosin (2 mg/ml) with trypsin (Worthington) was carried out at a weight ratio of protease to myosin of 1:100 in 0.6 M KCl, 40 mM imidazole–HCl, 1 mM dithioerythritol, 0.01% NaN3 (pH 7.0), 25°C. For the isolation and characterization of the soluble, tryptically fragmented myosin derivatives, the digestion was terminated by the addition of soybean trypsin inhibitor (twice the weight of trypsin); the digest was dialyzed overnight against 40 mM KCl, 40 mM imidazole, 0.1 mM dithioerythritol (pH 7.0). After centrifugation at 100 000 × *g* for 60 min at 4°C, the supernatant was submitted to DEAE-cellulose chromatography using a gradient of 40 mM – 0.6 M KCl in 40 mM imidazole–HCl, 0.1 mM dithioerythritol (pH 7.0). Another aliquot of this supernatant was fractionated on Sephacryl S-200 eluted with the imidazole buffer.

Tryptic proteolysis of gizzard chymotryptic HMM and papain S1 (2 mg/ml) was performed at 25°C in the interval time 0–35 min, in 40 mM imidazole–HCl, 40 mM KCl, 1 mM dithioerythritol, (pH 7.0); the weight ratios of the protease to HMM and S1 were 1:200 and 1:100, respectively. The tryptic fragments of S1 heavy chain were isolated as in [14]. Amino acid analysis was made as before [15] after hydrolysis for 24 h and 48 h at 110°C. SDS–Polyacrylamide gel electrophoresis was done in 5–18% polyacrylamide slabs [4]. The K^+- and Ca^{2+}-ATPase activities were determined as in [4]. The actin-activated Mg^{2+}-ATPase activity was measured in 35 mM KCl, 50 mM Tris–HCl (pH 8.0), 10 mM $MgCl_2$, 0.2 mM $CaCl_2$, 0.1 mM EGTA, 5 mM ATP, 1 mg/ml of actin and 0.100 mg/ml of myosin derivative, at 25°C.

3. RESULTS AND DISCUSSION

3.1. *Identification of the tryptic heavy chain fragments of gizzard myosin*

First, we identified all the tryptic fragments formed during a short period of digestion (15 min) under defined conditions (0.6 M KCl, pH 7.0, 25°C). Four major products of 29 kDa, 50 kDa, 66 kDa and 90 kDa were readily generated (fig. 1). The 29 kDa and 50 kDa bands together with their 75 kDa precursor peptide, came from the NH_2-terminus of the heavy chain. Their mobilities were similar to those corresponding to the 29 kDa, 50 kDa and 75 kDa tryptic peptides of skeletal S1

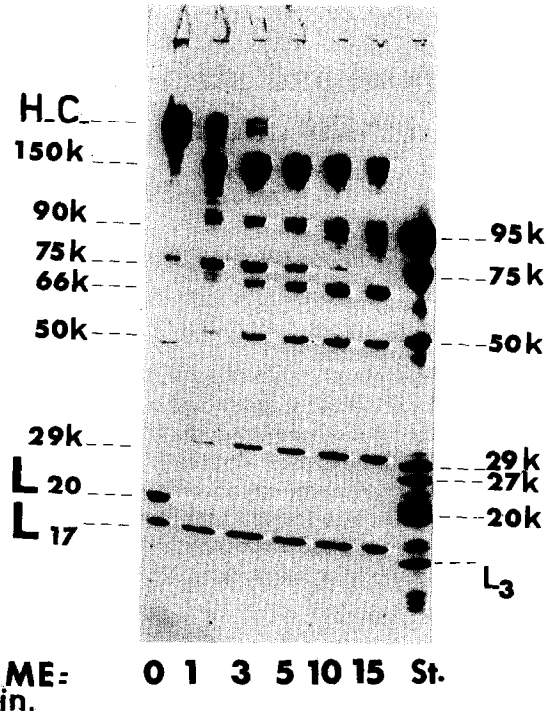

Fig. 1. The fragmentation of gizzard myosin heavy chains with trypsin analyzed by gel electrophoresis. For full experimental details, see the text. Protein markers (St) are: 95 kDa heavy chain of rabbit skeletal chymotryptic S1, its tryptic fragments, 75 kDa, 29 kDa, 27 kDa, 50 kDa, 20 kDa and L3 light chain.

heavy chain [15] used as markers. The 29 kDa and 50 kDa species were also produced in the digest and gizzard papain S1. The amino acid composition of the two isolated peptides showed similarities with the corresponding skeletal S1 peptides but the following differences were noticed (table 1). The content of the gizzard 50 kDa fragment in arginine was twice that found in the skeletal peptide while their contents in lysine are quite similar. Also, the former fragment contained much more tyrosine than the skeletal counterpart. The smooth 29 kDa peptide contained a significantly greater amount of Glx and Leu than the skeletal NH_2-terminal fragment.

The 66 kDa and 90 kDa fragments make up the tail part which, in agreement with [16], was first released as the 150 kDa band. Upon addition of F-actin to the digest and centrifugation, the supernatant contained only the 90 kDa band which was likely to be the subunit of the tryptic LMM; the 29

Table 1

Amino acid composition of the peptides derived from the head portion of gizzard myosin heavy chain[a]

Amino acid	Gizzard myosin tryptic fragments			Skeletal myosin tryptic fragments		
	50 kDa	29 kDa	26 kDa	50 kDa	27 kDa	20 kDa
Lys	30.0	21.5	14.2	(32.0)	(15.6)	(13.7)
His	7.0	5.8	4.0	(9.7)	(5.2)	(5.5)
Arg	20.0	7.4	12.3	(10.5)	(8.0)	(9.0)
Asx	45.0	26.2	21.8	(39.0)	(21.8)	(16.2)
Thr[b]	25.4	12.6	9.2	(25.7)	(12.8)	(8.7)
Ser[b]	22.6	18.4	6.0	(21.6)	(17.5)	(10.3)
Glx	61.0	32.8	28.4	(55.0)	(24.0)	(22.7)
Pro	15.0	10.3	6.8	(10.3)	(10.2)	(5.5)
Gly	30.8	17.4	15.0	(32.5)	(18.0)	(13.6)
Ala	32.7	17.8	14.0	(32.3)	(17.4)	(11.7)
1/2 Cys[b]	3.0	1.8	2.8	(4.0)	(1.8)	(2.2)
Val	20.8	12.0	10.4	(27.2)	(14.2)	(10.2)
Met	7.0	6.2	8.0	(11.0)	(7.0)	(4.2)
Ile	20.8	10.7	10.0	(24.0)	(12.0)	(7.7)
Leu	41.2	20.8	20.0	(36.6)	(12.7)	(15.5)
Tyr	29.7	8.0	5.2	(18.2)	(9.0)	(3.5)
Phe	21.8	8.0	12.2	(24.0)	(8.7)	(7.0)

[a]The data shown are averaged values from at least 6 determinations on 24 h and 48 h hydrolysates. The values are expressed as residues/mol peptide assuming the indicated apparent masses. The peptides were isolated from tryptic digests of papain S1

[b]Not corrected for hydrolytic losses

kDa, 50 kDa and 66 kDa species together with the 17 kDa light chain remained in the pellet combined with actin. The 66 kDa peptide included the intact S1–S2 junction; addition of papain to the tryptic digest led to the selective scission of this fragment into 2 components of 26 kDa and 37 kDa (fig. 2). The 26 kDa peptide was also produced by digestion of gizzard S1 with trypsin, together with the 29 kDa and 50 kDa peptides. It corresponds to the COOH-terminal segment of the S1 heavy chain. Its amino acid composition is depicted in table 1 in comparison with that known for the corresponding 20 kDa fragment from skeletal S1 [15].

3.2. *Tryptic sensitivity of the head-rod junction of gizzard myosin heavy chain*

According to the above results, the S1–S2 link within the 66 kDa fragment is apparently resistant to trypsin under the conditions employed. However, to understand more fully the mode of action of the protease on this region, we investigated the fragmentation of chymotryptic HMM with trypsin during a longer period of reaction (35 min). The digestion of the 130 kDa HMM heavy chain gave rise to the 29 kDa and 50 kDa fragments together with a 60 kDa peptide which, of course, must contain the S1–S2 junction. However this entity was progressively degraded and it disappeared after 35 min reaction without the concomitant formation of obvious breakdown products. The susceptibility of the 60 kDa region to trypsin was confirmed by a new analysis of the 15 min tryptic digest of the parent myosin. This digest was submitted to chromatography on DEAE-cellulose and to filtration over Sephacryl S-200.

Both fractionation procedures led to the separation of two protein species 'a' and 'b' (fig.4A); the amount of the minor fraction 'a' was 15–20% of the starting protein digest. Gel electrophoresis of

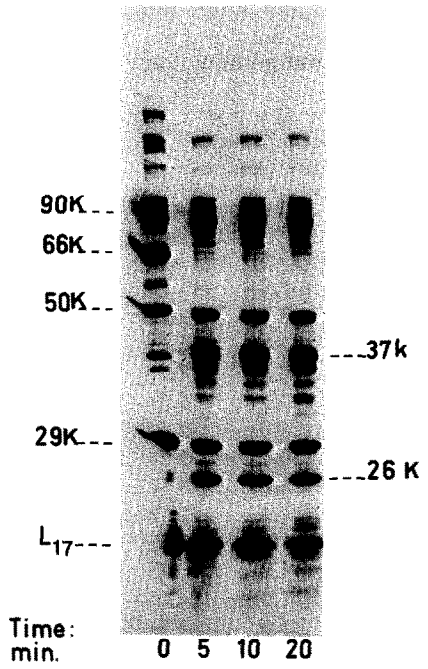

Fig. 2. Identification of the tryptic 66 kDa segment of the heavy chain by selective papain cleavage at the S1-S2 junction. A 15 min tryptic digest of myosin was supplemented with 1 mM EDTA and was incubated at papain/myosin of 1:1000 (w/v) (pH 7.0) 25°C.

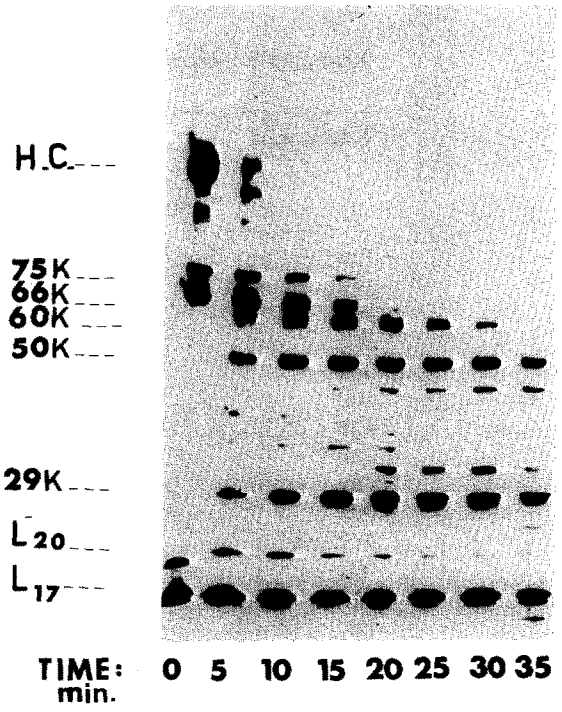

Fig. 3. Time-course of the fragmentation of the heavy chain of gizzard chymotryptic HMM with trypsin. For full experimental details, see the text.

each isolated fraction (fig.4B) showed consistently in peak 'a' the presence of only the 29 kDa and 50 kDa heavy chain peptides together with the 17 kDa light chain. This pattern was identical to that observed in fig.3 for the 35 min tryptic digest of chymotryptic HMM. In contrast, protein 'b' contained intact 66 kDa peptide in addition to the 3 other constituents. The data suggest that species 'a' is a S1-like species resulting from a slow breakdown of the tryptically labile 66 kDa fragment; the COOH-terminal 26 kDa material of its heavy chain was presumably degraded into small peptides non-detectable on the gel.

The elution of the major peak 'b' near the void volume of the Sephacryl S-200 column and the nature of its peptide composition are consistent with the idea that it corresponds to tryptic HMM whose heavy chain is a complex of the 3 fragments, 29 kDa, 50 kDa and 66 kDa, held together by non-covalent forces. This purified protein preparation, referred to as (29 kDa-50 kDa-66 kDa)-HMM, displayed (Mg^{2+}/Ca^{2+})- and K^+-dependent ATPase activities corresponding to 0.015, 0.22 and 0.40 $\mu M\ P_i.min^{-1}.mg^{-1}$, respectively; similar values were also found for the protein 'a'. Since the degraded 26 kDa peptide can remain attached within this fraction, we attempted to assess its presence by performing the digestion on gizzard myosin labeled with the fluorophore 1,5-IAEDANS which, in skeletal myosin, reacts selectively with SH1 present in this region [17]. But this attempt failed as the reagent reacted also with the 50 kDa peptide and 17 kDa light chain. The incorporation of other thiol reagents into these components of gizzard S1 was observed in [18].

3.3. *Actin-binding properties of the tryptic HMM and S1 species*

The HMM and S1 derivatives were not activable by actin; we have investigated the extent of their binding to skeletal muscle F-actin by measuring the inhibitory effect of actin on their K^+-ATPase activity [19,20]. The addition of increasing concentrations of actin (molar ratio actin/head = 0-2) in-

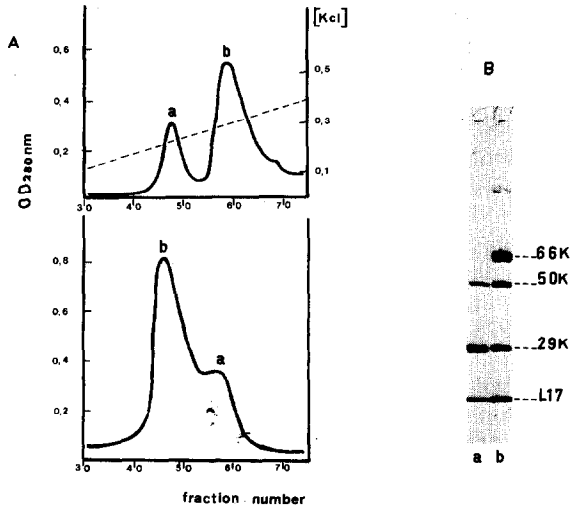

Fig. 4. Isolation and identification of the tryptic HMM and S1 species from gizzard myosin. (A) Two equal portions of the 15-minutes tryptic digest of myosin (50 mg each) were fractionated on a column of DEAE-cellulose (1.60×33 cm) (upper) and on a column of Sephacryl S-200 (1.50×1.70 cm) (lower), as in section 2; (B) Samples of the pooled fractions 'a' and 'b' from the ion-exchange chromatography column were analyzed by gel electrophoresis (lanes a and b, respectively).

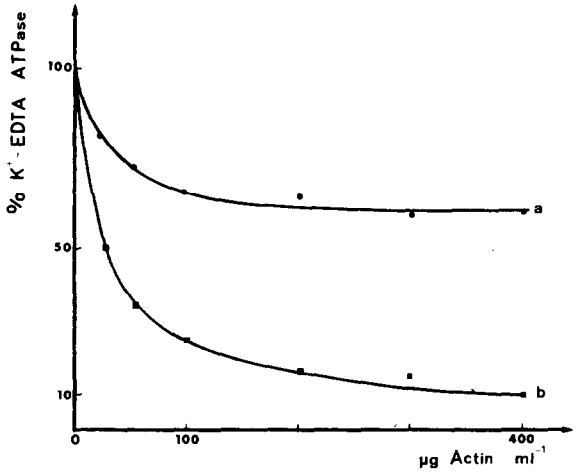

Fig. 5. Influence of actin on the K^+-ATPase of gizzard tryptic HMM (b) and S1 (a). Conditions: 200 μg enzymes/ml and 0–10 μM actin in 1 M KCl, 5 mM EDTA, 2.5 mM ATP (pH 7.5), 25°C.

fluenced the activity of the HMM entity much more effectively than that of the fraction 'a' (fig.5). At a high actin concentration the inhibition of the latter derivative reached maximally 35% while the HMM preparation was inhibited to about 95%. The molar ratio actin/head producing 35% inhibition of the K^+-ATPase was 1 and 0.1 for the fraction 'a' and the HMM species, respectively. Sedimentation of the fraction 'a' in the presence of F-actin in 40 mM imidazole–HCl (pH 7.0) 4°C, brought down little or no protein material. The data suggest that while the tryptic HMM seems to bind completely to F-actin, the species 'a' exhibits a weak affinity for actin and that the structural integrity of the 26 kDa region is essential for the maintenance of the actin-binding properties of the smooth myosin head heavy chain.

Finally, fig.6 shows that the Mg^{2+}-ATPase of the tryptic HMM could be activated 5–6-fold by actin upon cleavage of the 66 kDa segment by papain under the conditions reported in fig.2. Furthermore, the time course of this activation was nearly the same for tryptic HMM which lacks the 20 kDa light chain and for the regulated chymotryptic HMM which contains an intact 20 kDa subunit. Since gizzard papain S1, with and without phosphorylatable light chain, is fully activable by actin [21], the inhibited state of the light-chain-depleted tryptic HMM seems to be imposed mainly

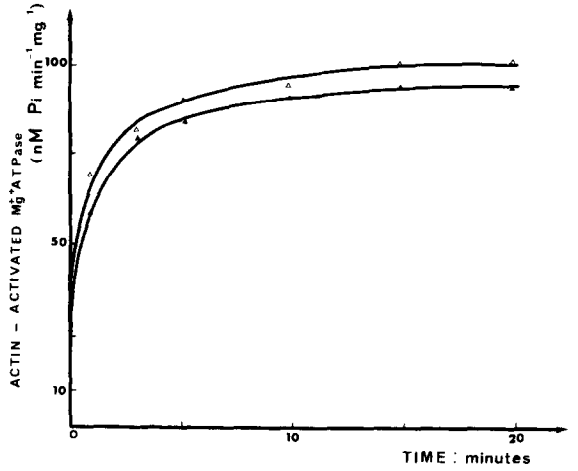

Fig. 6. Derepression of the actin-activated Mg^{2+}-ATPase of tryptic (△) and chymotryptic HMM (▲) by cleavage of the 66 kDa fragment with papain. Enzymes (6 μM) were incubated with papain as in fig. 2. At various times, aliquots were assayed for actin-activated ATPase.

by the integral 66 kDa segment. Thus, this region could contribute directly to the regulation of the gizzard actomyosin ATPase by inhibiting this activity [6] when the light chain is not phosphorylated.

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