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Crosslinking of trypsin digested acto-heavy meromyosin as a probe of the affinity of the two myosin heads to actin

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The interaction of the two heads of the myosin molecule with actin was studied by tryptic digestion of HMM in the presence of actin, followed by crosslinking the two nicked heavy chains with Nbs_2 at the S2 region. In view of the protection by actin of the 50/60 kDa junction against proteolysis, the percentage of the heads interacting with actin was estimated from the proportion of the 110 kDa to the 60 kDa digestion product. Under conditions such that about 50% of HMM heads were protected by actin (at an actin to HMM head molar ratio of 1:1 in the absence of nucleotide, or 3:1 in the presence of 5 mM ADP), the crosslinking of the digestion products yielded a 230 kDa (110+110 kDa), 125 kDa (60+60 kDa) and 175 kDa (60+110 kDa) species. Since the latter should be the only crosslinking product when only one head of HMM molecule is protected by actin, it is concluded that there is no preferential binding of one of the two HMM heads to actin in the presence of ADP or at equimolar actin to myosin heads ratio.

Heavy meromyosin; Actin-myosin interaction; Disulfide crosslinking; Tryptic proteolysis

1. INTRODUCTION

It is known that during muscle contraction the affinity of the myosin heads for actin undergoes cyclic changes, modulated by the binding and hydrolysis of ATP. However, the functional significance of the double-headed structure of the myosin molecule remains unexplained.

A few years ago the tryptic digestion method was introduced to studies of the interaction of the myosin heads with actin in myofibrils under rigor conditions [1]. It was based on the observation that limited tryptic proteolysis of myosin S1 results in the formation of three relatively stable heavy chain fragments of 25, 50 and 20 kDa [2–4], and that the addition of actin changes the digestion pattern by protecting the 50/20 kDa junction against tryptic attack [3,4]. Application of this method to the investigation of the influence of ATP analogues on the state of crossbridges in myofibrils showed that in the presence of MgADP about 50% of the myosin heads had significantly lower affinity to actin than the rest of the heads [5]. Similar differentiation of the affinity to actin, detected by the same proteolytic technique, was observed for HMM (but not for S1) in the absence of nucleotide, at an actin to HMM head molar ratio of 1:1 [6]. The results of these studies were interpreted in terms of possible different affinity for actin of the two heads of the myosin molecule, a feature that had been suggested earlier based on some other studies on muscle fibers [7,8] and on isolated myosin and its

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Abbreviations: HMM, heavy meromyosin; S1, myosin subfragment 1; S2, myosin subfragment 2; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; Nbs_2, 5,5′-dithiobis-2-nitrobenzoate; AP_5A, P^1P^5-di-(adenosine-5′)-pentaphosphate

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subfragments [9–13]. However, no clear evidence for the differential interaction of the two myosin heads with actin has been presented so far.

In the present work, the behaviour of the two heads of the myosin molecule was tested by chemical crosslinking the two heavy chains of HMM at the S2 region following the tryptic digestion in the presence of actin and by analysing the crosslinking products by SDS-PAGE. The results show that there was no preferential binding to actin of one of the two HMM heads when about 50% of the heads were bound either at the equimolar actin to myosin heads ratio or at higher ratios in the presence of ADP.

2. MATERIALS AND METHODS

Myosin was prepared from rabbit fast skeletal muscles, and HMM was obtained by digestion of myosin with chymotrypsin [14]. Tryptic digestion of HMM (3 mg/ml) was carried out at the enzyme to substrate ratio of 1:50 (w/w) in 100 mM KCl, 20 mM Hepes, pH 7.6, and 5 mM $MgCl_2$, at various concentrations of actin, at 24°C. Samples digested in the presence of ADP (5 mM) contained 0.4 mM AP_5A to inhibit myokinase possibly present as a contaminant. The digestion was terminated by addition of soybean trypsin inhibitor twice the weight of trypsin. After the digestion, HMM was oxidized by incubation with 2 mM Nbs_2 for 7 min. The reaction was stopped by addition of 1% SDS containing 10 mM iodoacetate (final concentrations), and the excess reagents were removed by exhaustive dialysis against 1% SDS. SDS-PAGE of HMM before and after oxidation was carried out according to [15] using 6% acrylamide, in the presence and absence of 2-mercaptoethanol, respectively. For identification of the crosslinking products of the oxidized HMM, the slices of the gels were cut off and applied on the top of the SDS-gel slabs prepared according to [16], using 6% stacking and 10% separating gels. To reduce disulfide bonds, a warm 2% agarose containing 3% 2-mercaptoethanol was added on the top of the gel before each run. The molecular masses of the crosslinking products were estimated by comparing electrophoretic mobilities to those of actin oligomers obtained by crosslinking of actin with glutaraldehyde [17].

3. RESULTS AND DISCUSSION

The HMM molecule comprises the two myosin heads and the S2 portion of the rod. Since the presence of divalent cations protects the S1/S2 junction against tryptic attack [18], the digestion of the heavy chain of HMM in the presence of magnesium results in the formation of the 25, 50 and 60 kDa fragments, the latter composed of S2 and the C-terminal domain of the head portion of the heavy chain (fig.1A). Addition of actin at a molar ratio to HMM head of 3:1 almost fully protected the 50/60 kDa junction against tryptic attack as expected, leading to the formation of the 25 and a 110 kDa fragment (fig.1B). The additional presence of a 72 kDa peptide on the gel indicates that, in spite of the addition of divalent cation, HMM was partly cleaved also at the S1/S2 junction. When the ratio of actin to HMM head was lowered to 1:1 or when ADP was additionally included in the digestion mixture, roughly equal amounts of the relatively stable 60 and 110 kDa fragments were formed during 60 min digestion (fig.1C,D). 5 mM ADP was used since lower con-

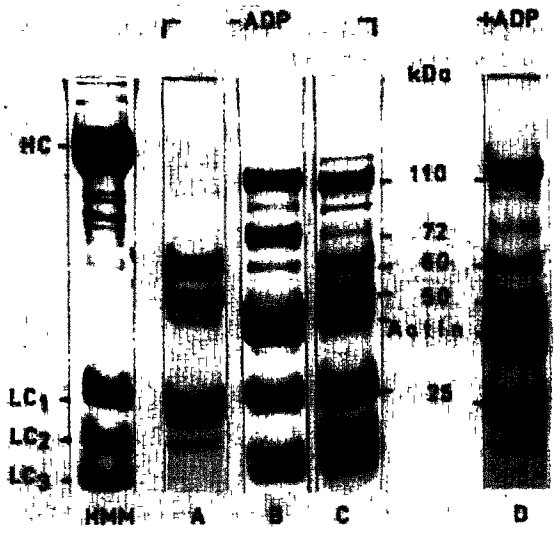

Fig.1. SDS-PAGE of HMM before and after tryptic digestion. HMM was digested for 30 (A) or 60 min (B) as described in section 2, in the absence or presence of ADP as indicated. Actin was absent (A) or present during digestion at a molar ratio to HMM head of 3:1 (B,D) or 1:1 (C). SDS-PAGE was carried out in the presence of 2-mercaptoethanol according to [15].

centrations were not sufficient to markedly influence the proteolytic susceptibility of the myosin heads in synthetic acto-myosin or acto-S1 systems [5,19]. The relatively high amount of the peptide with the molecular mass of 25 kDa is consistent with the observation that the 60 kDa fragment was partly split to S2 (partly masked by actin on the gel) and C-terminal domain of the head; the latter is likely to have the mass close to that of the N-terminal 25 kDa fragment [20].

To test the assumption that one of the two heads of the myosin molecule is preferentially protected by actin, the digestion products were crosslinked with Nbs_2 which is known to form disulfide bonds in the S2 region of the myosin molecule [21,22]. The subsequent addition of SDS leads to the dissociation of uncrosslinked proteolytic fragments of HMM. The expected crosslinking products are shown in fig.2.

The products of the reaction of HMM with Nbs_2 were analyzed by SDS-PAGE before and after reduction of the disulfide bonds (fig.3). Crosslinking of HMM digested without actin (fig.3A) yielded a 125 kDa species composed of two 60 kDa fragments as expected (see I in fig.2). The other major peptides seen on the gel are 25 and 50 kDa domains of the myosin head and some uncrosslinked 60 kDa fragments.

When the cleavage of the HMM heads at the 50/60 kDa site was almost fully inhibited by the presence of actin in excess to HMM, a 230 kDa product, identified as the two crosslinked 110 kDa fragments, was formed (fig.3B, cf. II in fig.2). A 140 kDa species comprising the 110 and 37 kDa (S2) fragments was also present (see IIa in fig.2).

When 50% of HMM heads are cleaved at the 50/60 kDa site, the 175 kDa species (60+110 kDa) should be the only crosslinking product if only one HMM head is protected by actin (see III in fig.2). As shown in fig.3C,D when the actin to HMM ratio was lowered or when ADP was present during the digestion, the 175 kDa product was accompanied by two other major species, the 125 and 230 kDa ones. Changing the pH to 7 and increasing the KCl concentration to 200 mM during the digestion of HMM at the equimolar actin to HMM heads ratio to reproduce the conditions used in [6] did not essentially influence the pattern of the crosslinked fragments (not shown). The additional presence of a minor 100 kDa product, composed of the 60 and 37 kDa fragment, seems to result from some cleavages at the S1/S2 junction (see Ia in fig.2).

It could be argued that the estimation of the concentration of the proteolytic fragments is only semi-quantitative and the actual extent of the tryp-

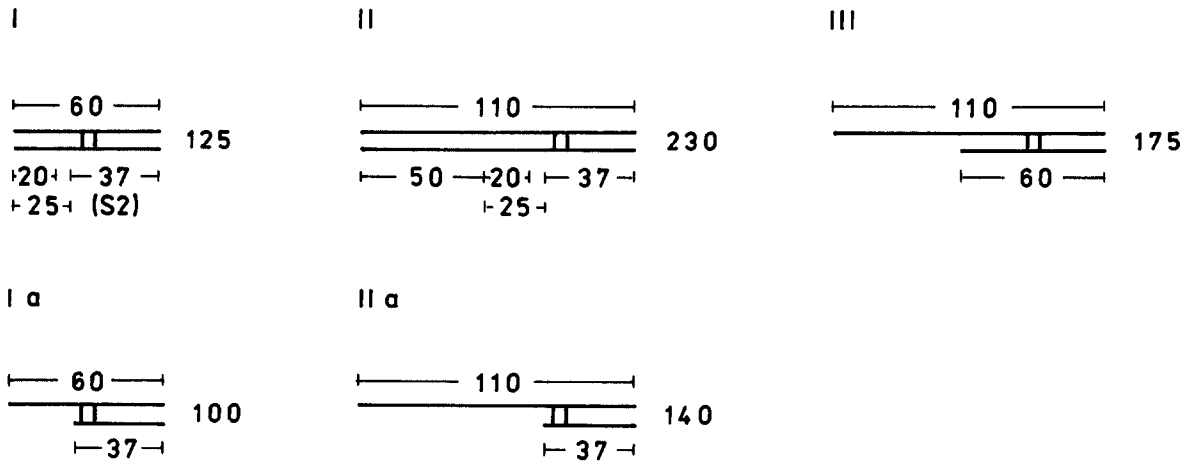

Fig.2. Expected products of the crosslinking of the tryptic peptides of HMM. I, digestion in the absence of actin; II, protection by actin of both HMM heads; III, protection by actin of one HMM head against proteolysis. Ia and IIa show crosslinking products when one S1/S2 junction in HMM molecule is cleaved; these two products can also be formed in III. The numbers represent the size (in kDa) of the various fragments. Molecular masses are those estimated from the electrophoretic mobilities of the peptides.

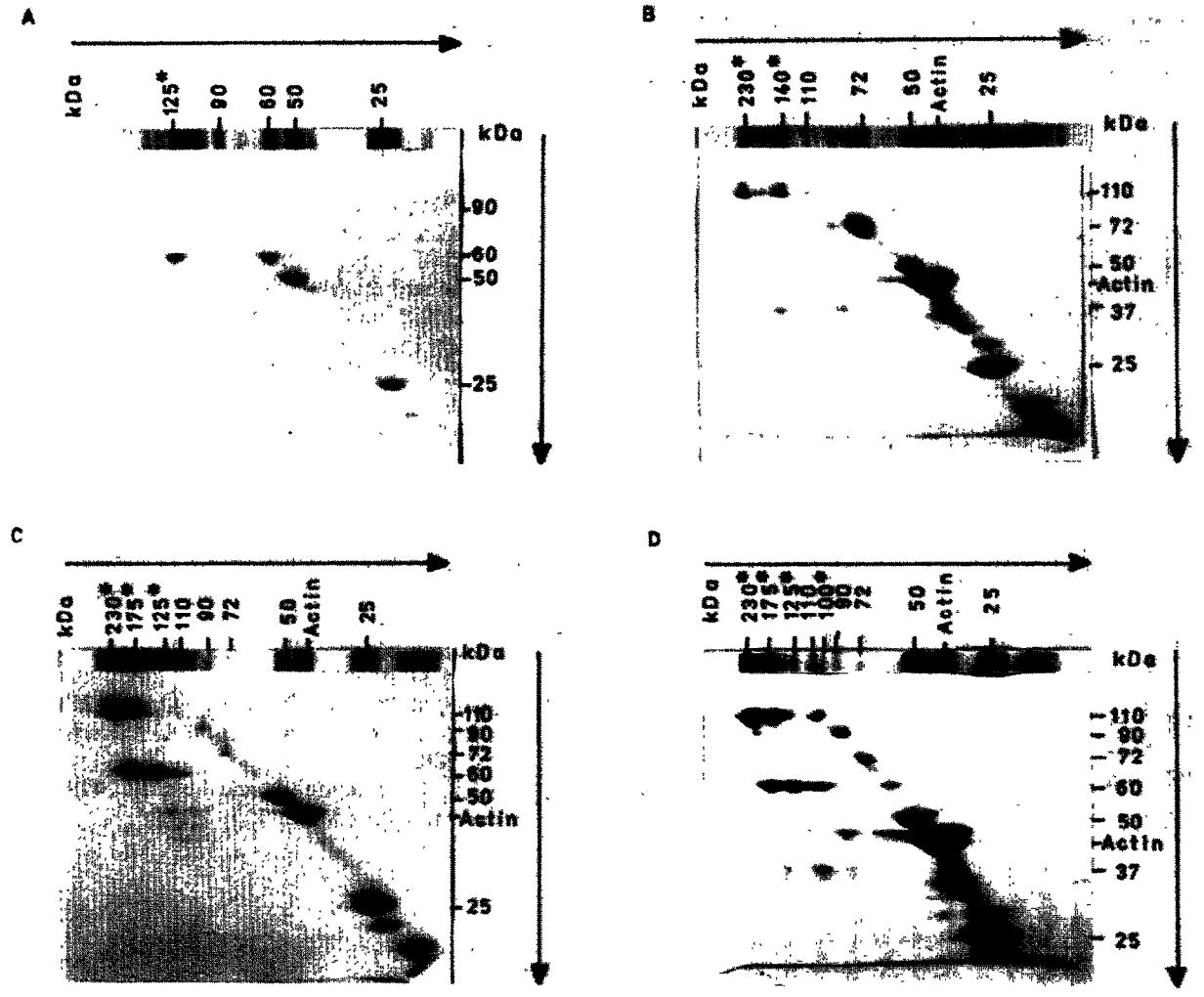

Fig.3. Analysis of the crosslinking products. The upper part of each slab shows the crosslinking products of the tryptic peptides of HMM separated by SDS-PAGE in the absence of reducing agents; the slabs show the subsequent separation in the presence of 2-mercaptoethanol as described in section 2. A, B, C, D correspond to A, B, C, D in fig.1 after reaction with Nbs_2. Molecular masses of the crosslinking products are marked with asterisks.

tic cleavage at the 50/60 kDa site was not exactly 50%. Nevertheless, in the case of differential reactivity of the two heads, the 175 kDa product might be accompanied by either the 230 kDa (at less than 50% digestion) or the 125 kDa species (at more than 50% digestion), but never by both of them. Thus it can be concluded that under the conditions used in this experiment the accessibility to trypsin was not limited to one head of HMM. Consequently, the binding of one head of HMM to actin does not seem to interfere with the binding of the other one.

It should be noted that the differential behaviour of the myosin heads with respect to their interaction with actin has been attributed by some investigators to more complex systems like myofibrils, but not to the purified proteins in solution [5,23]. In such a case, possible functional difference between the two heads of the myosin molecule, not observed in the present study on

HMM, could be imposed by the supramolecular organization of the proteins in the myofibrillar structure.

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