Effect of the integrity of the myofibrillar structure on the tryptic accessibility of a hinge region of the myosin rod

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Limited proteolysis has been used to study the influence of actin, in the absence or presence of regulatory proteins of the thin filament (tropomyosin and troponin), as well as that of the myofibrillar structure on the tryptic cleavage of the heavy meromyosin (HMM)/light meromyosin (LMM) hinge region in myosin heavy chain. Cleavage at the HMM/LMM hinge is almost absent in myofibrils, whereas this hinge is accessible to tryptic digestion in actomyosin, in native thin filaments attached to myosin and in myosin heavy chain alone. This observation indicates that it is the myofibrillar structure which profoundly affects the tryptic accessibility of this specific hinge region of myosin. This provides a good example of the manner by which a highly organized supramolecular structure might affect the chemical properties of a specific site in a macromolecule.

Myofibrillar structure *Myosin hinge region* *Tryptic proteolysis*

1. INTRODUCTION

Limited proteolysis has been widely used as a tool in the study of myosin structure and myosin-actin interaction. There are two hinge regions in myosin: one between the heads and the rod (S-1/S-2 junction) and the other in the rod (HMM/LMM junction). Cleavage at both hinges can be performed with a number of proteolytic enzymes and is known to depend on the concentration of divalent cations and ionic strength [1–3]. The heavy chain of S-1 can be further cleaved by trypsin to 3 fragments, whose molecular masses are as follows: 25, 50 and 20 kDa, starting from the NH_2 terminus [4,5]. There is mounting evidence that these fragments are distinct domains of S-1 [6–8]. The cut between the 50 and 20 kDa domains (75 kDa from the NH_2 terminus) is blocked in the presence of actin [5]. A similar result, i.e. the blocking of the tryptic cut at a site 75 kDa from the NH_2 terminus of myosin heavy chain, has been obtained with myofibrils in rigor, that is when the myosin heads are tightly attached to actin [9–11], whereas in relaxation, when the myosin heads are detached from actin, the above site is exposed to mild tryptic attack. Recently, we have studied the limited tryptic digestion of myosin in myofibrils under rigor conditions and compared it to the digestion of isolated myosin. One of the main differences was that the cleavage at the HMM/LMM hinge in the rod segment of myosin was reduced in myofibrils relative to isolated myosin. It seemed to us of interest to study this question in greater detail, in particular because of the significance of the HMM/LMM hinge region in the molecular mechanism of contraction, as

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Abbreviations: DTT, 1,4-dithiothreitol; HMM, heavy meromyosin; 1,5-IAEDANS, 5-iodoacetamidoethyl-aminonaphthalene-1-sulfonic acid; LMM, light meromyosin; S-1, heavy meromyosin subfragment-1; S-2, heavy meromyosin subfragment-2

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assumed by Harrington and his co-workers [12,22,23]. Here, we studied the effects of actin, of the regulatory proteins of the thin filament (tropomyosin and troponin), as well as those of the myofibrillar structure on the tryptic cleavage of the HMM/LMM hinge region. Our results indicate that it is the myofibrillar structure which profoundly affects the tryptic accessibility of this specific hinge region of myosin. This provides an excellent example of the manner by which a highly organized supramolecular structure might affect the chemical properties of a specific site in a macromolecule.

2. MATERIALS AND METHODS

2.1. *Materials*

α-Chymotrypsin, trypsin, soybean trypsin inhibitor and 1,5-IAEDANS were purchased from Sigma. All other chemicals were of analytical grade. Water was glass double-distilled.

2.2. *Preparation of myofibrils and proteins*

Myofibrils were prepared from glycerinated fibers of rabbit psoas muscle as described [3], except that they were finally suspended in rigor solution containing 80 mM KCl, 10 mM Na-phosphate buffer, pH 7.0, 2 mM EGTA, 5 mM $MgCl_2$. The protein concentrations of myofibrillar suspensions were determined by the biuret method. Myofibrils were digested on the day of preparation to avoid proteolysis by endogenous proteases. Myosin was prepared according to Tonomura et al. [13]. Native thin filaments were prepared by the method of Knight and Lovell [14]. Actin was prepared as described by Spudich and Watt [15]. Myosin was mixed with F-actin or native thin filaments at a molar ratio myosin:actin of 1:2 in rigor buffer.

2.3. *Fluorescent labeling of myosin with 1,5-IAEDANS*

Myosin (30 mg/ml) in 0.6 M KCl-3 mM NaH_2PO_4 (pH 7.0) was reacted in the dark with a 10-fold molar excess of 1,5-IAEDANS for 1 h at 0°C [16]. Unreacted 1,5-IAEDANS was quenched by the addition of 10 mM DTT and then removed by dialysis.

2.4. *Tryptic digestions*

All digestions were carried out at 25°C. Myofibril concentration was fixed at 2 mg/ml. Tryptic digestions were carried out at a ratio of trypsin to total myofibrillar protein of 1:40 (w/w). Digestions were terminated by the addition of a 3-fold excess of soybean trypsin inhibitor over trypsin (w/w).

2.5. *Quantitative SDS gel electrophoresis*

Polyacrylamide gel electrophoresis in the presence of SDS was carried out according to Laemmli [17] using a gradient resolving gel (5–15% acrylamide). The distribution of fluorescence among the 1,5-IAEDANS-labeled proteins was monitored by photographing the gels under UV illumination. The same gels were thereafter stained with Coomassie brilliant blue R. The molecular masses of the proteolytic fragments were determined using the Pharmacia calibration kit markers: phosphorylase *b* (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa),

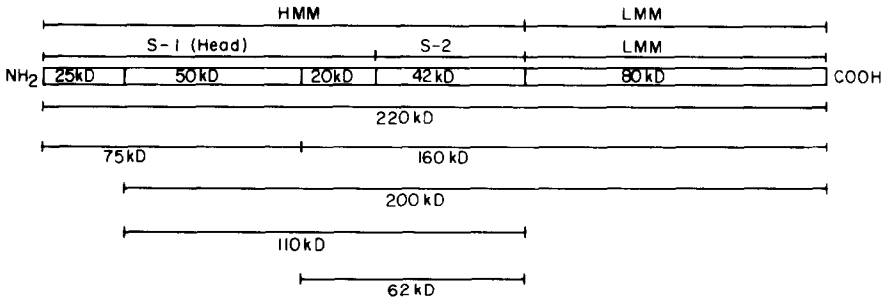


Fig.1. Linear representation of the myosin heavy chain and tryptic fragments obtained under various conditions. The drawing indicates the origin, the alignment in the heavy chain and the molecular mass (in kDa) of each peptide fragment mentioned in the text.

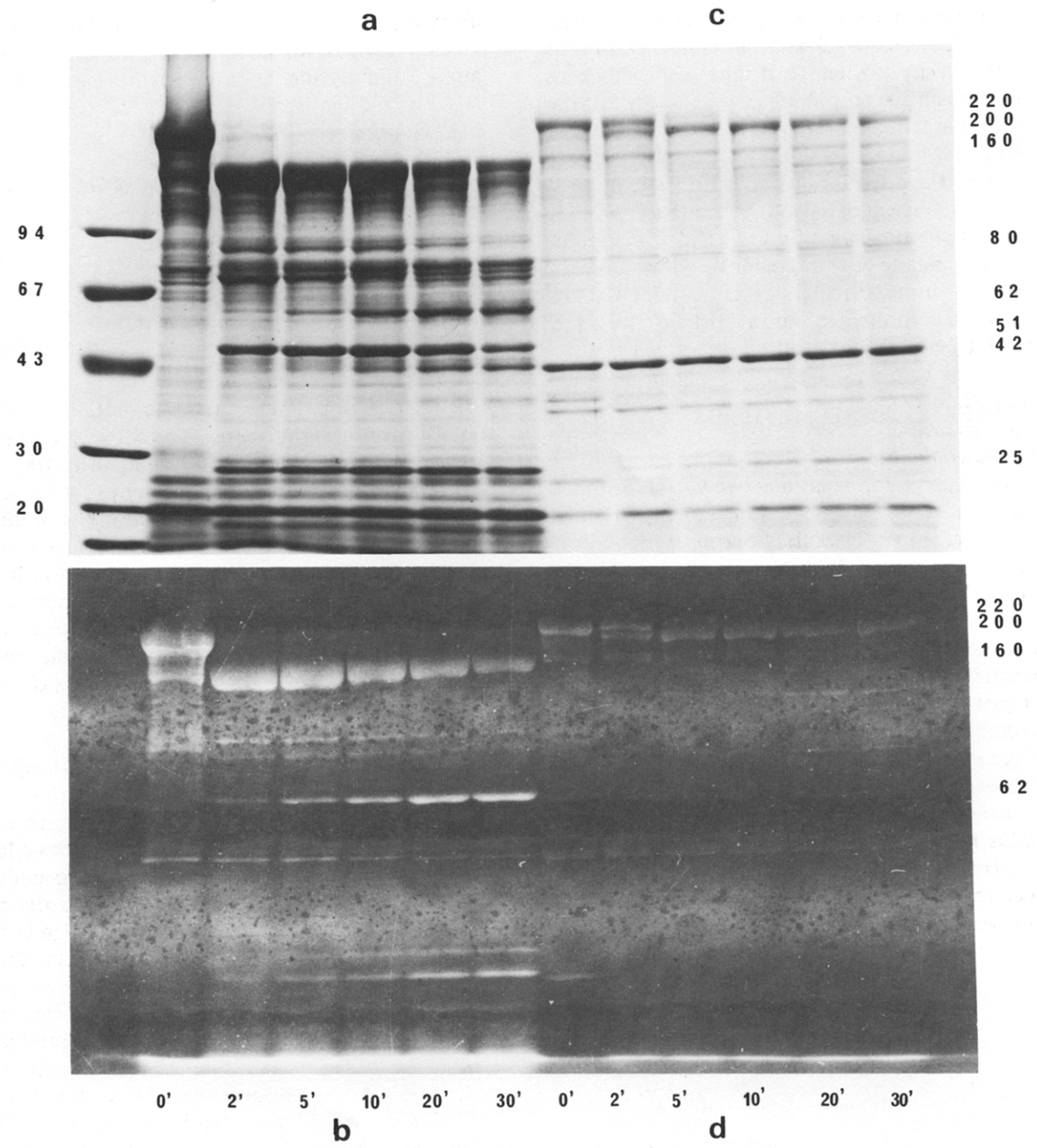


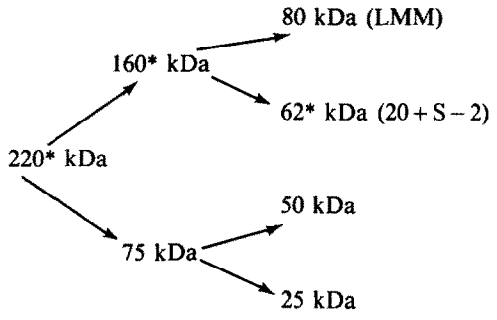
Fig.2. SDS-polyacrylamide gel electrophoretic patterns of tryptic digests of myosin (a,b) and myofibrils (c,d) in rigor at 25°C as a function of time. Panels: (a,c) Coomassie blue-stained protein bands, (b,d) fluorescence of the 1,5-IAEDANS-labeled protein bands. The molecular masses of the marker proteins are shown on the left of panel a. The positions of actin, of the heavy chain of myosin and of its tryptic fragments are shown on the right of panel c and some of them on the right of panel d. Digestion times (in min) are indicated under each lane of panels b and d. The corresponding lanes in panels a and c are somewhat off-position, due to gel swelling under the conditions of staining.

carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa) and α-lactalbumin (14.4 kDa). The intrinsic markers were: myosin heavy chain (220 kDa) and actin (42 kDa).

3. RESULTS

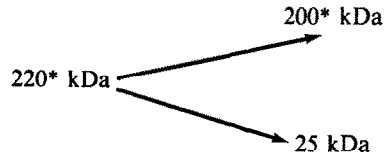
3.1. *Comparison of the digestion of purified myosin with that of myofibrils*

A schematic representation of the tryptic fragments of myosin observed in this work is shown in fig.1. Tryptic digestion of myosin under the conditions of rigor resulted in the formation of the 160 and 75 kDa fragments (fig.2a). Within 2 min all the myosin heavy chain had been degraded to produce these fragments. The 75 kDa fragment was readily further degraded to 50 and 25 kDa fragments. The digestion of the 160 kDa fragment was much slower and produced fragments with molecular masses of 80 and 62 kDa. To identify the fragments we specifically labeled the SH_1 group of the myosin heavy chain with a fluorescent dye, 1,5-IAEDANS. This SH_1 group is localized on the 20 kDa fragment of the myosin heavy chain. In fig.2b the tryptic digestion of myosin (same as shown in fig.2a) was followed on the basis of the change in the fluorescence pattern of the labeling dye. It is clearly seen that the dye was incorporated into the 160 and 62 kDa fragments, which indicates that both contained the 20 kDa fragment. The digestion of myosin can be represented as follows (in all schemes the asterisk indicates the fragment which is labeled with the fluorescent 1,5-IAEDANS).



Scheme 1

For comparison fig.2c and d shows the electrophoretic pattern of the tryptic digestion of myofibrils at full overlap: it is clear that myosin heavy chain was rapidly digested to the 200 and 25 kDa fragments. The following (scheme 2) represents the tryptic digestion of myosin in myofibrils.



Scheme 2

The fact that the pattern did not essentially change after a 10 min digestion indicates that the fragments formed were rather resistant to further tryptic attack. It seems that both sites, at 75 kDa from the N-terminus and at the HMM/LMM junction, which were exposed to trypsin in purified myosin, became inaccessible in the myofibrillar structure. The site at 75 kDa from the N-terminus is known to be protected from tryptic hydrolysis by actin [9]. However, the effect of actin on the trypsinolysis of the HMM/LMM junction is less well understood.

3.2. *Effect of F-actin on the digestion of purified myosin*

Fig.3a and b shows the tryptic digestion of actomyosin obtained when purified F-actin was added to purified myosin in a 2:1 molar ratio under rigor conditions. It is quite obvious that actin inhibited the cleavage at the 75/160 kDa site in both actomyosin (fig.3a) and myofibrils (fig.2c), and resulted in the formation of the 200 kDa fragment. However, the latter fragment was further cleaved into 110 kDa (HMM) and 80 kDa (LMM) peptides in actomyosin, whereas it remained resistant to tryptic attack in myofibrils. It became clear from the comparison of the tryptic digestion of actomyosin with that of myosin (figs 3a and 2a), that actin did not inhibit the cleavage at the HMM/LMM hinge, but even accelerated it (the 200 kDa fragment disappeared faster from the electrophoretic pattern of actomyosin than the 160 kDa fragment from that of myosin). The 110 kDa fragment formed during the actomyosin digestion was not very stable and it was slowly further degraded to the 50 and 62 kDa peptides. The

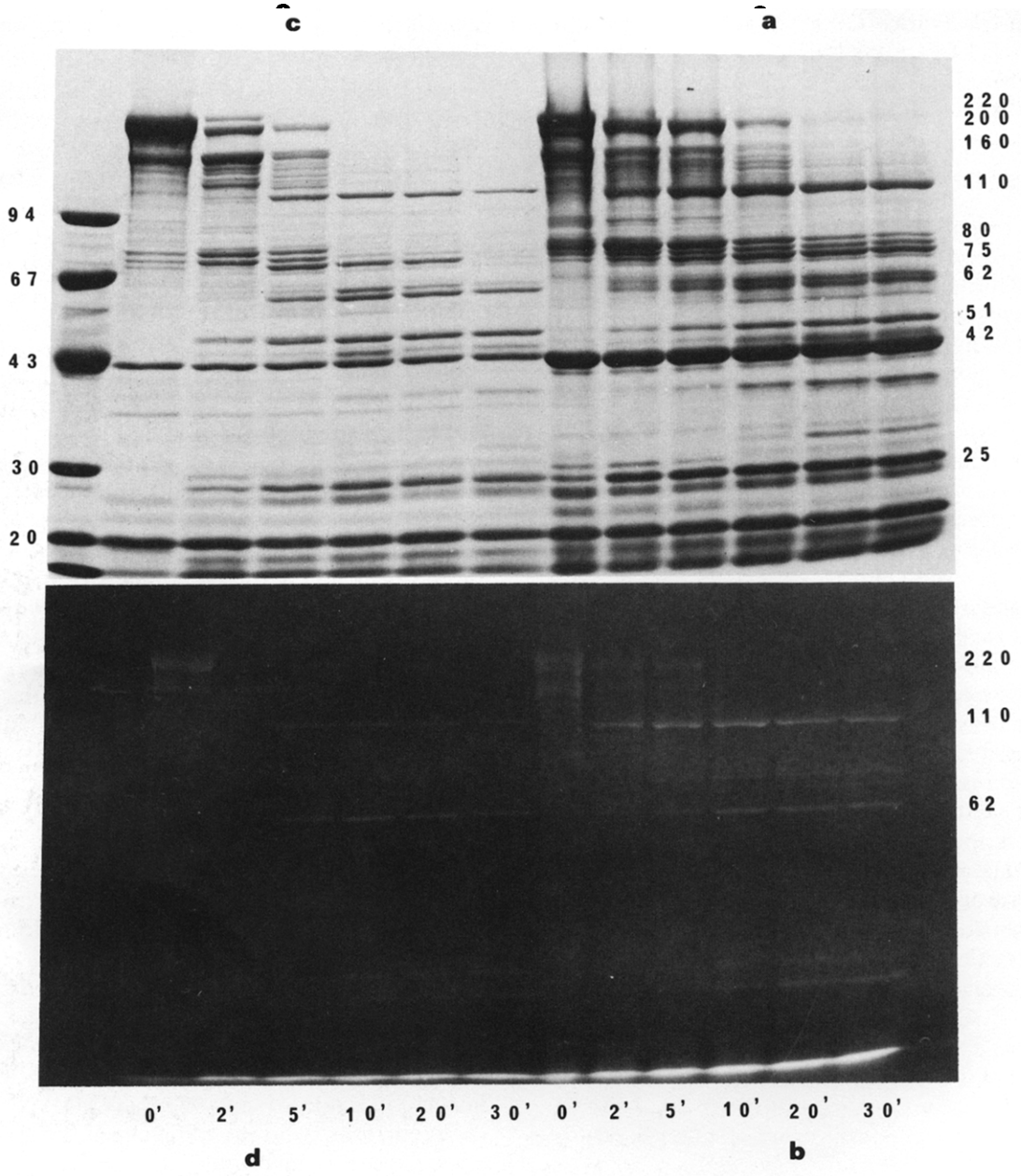
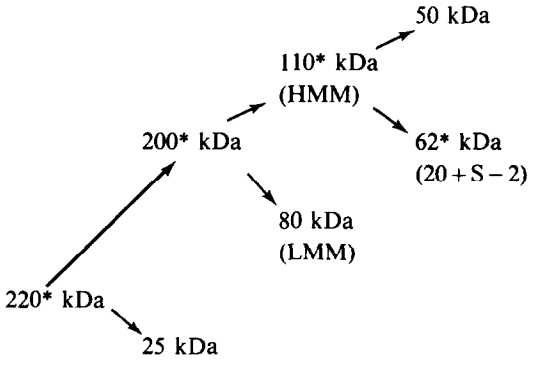


Fig.3. SDS-polyacrylamide gel electrophoretic patterns to tryptic digests of actomyosin (a,b) and native thin filaments (c,d) added to myosin in rigor at 25°C as a function of time. Panels: (a,c) Coomassie blue-stained protein bands, (b,d) fluorescence of the 1,5-IAEDANS-labeled protein bands. The molecular masses of the marker proteins are shown on the left of panel c. The positions of actin, of the heavy chain of myosin and of its tryptic fragments are shown on the right of panel a and some of them on the right of panel b. Digestion times (in min) are indicated under each lane of panels b and d. The corresponding lanes in panels a and c are somewhat off-position, due to gel swelling under the conditions of staining.

latter is composed of the C-terminal 20 kDa fragment of S-1 and of S-2.

The digestion pattern of actomyosin is illustrated in the following (scheme 3).



Scheme 3

3.3. *Effect of native thin filaments on the digestion of purified myosin*

The aforementioned results clearly indicate that the protection of the HMM/LMM hinge from tryptic cleavage in myofibrils is not due to the attachment of actin to myosin in rigor. To test another possibility, namely that this protection could be due to the presence of the regulatory proteins troponin and tropomyosin in myofibrils, purified myosin was attached to isolated native thin filaments, which, in addition to actin, also contain troponin and tropomyosin. Fig.3c and d shows the tryptic digestion of myosin attached to native thin filaments under rigor conditions. The digestion pattern of myosin attached to native thin filaments is practically indistinguishable from that of 'synthetic' actomyosin (fig.3a). Thus, it seems that the protection of the HMM/LMM hinge of the myosin rod from proteolysis in myofibrils is not caused by the attachment of myosin to actin in the absence or presence of regulatory proteins.

4. DISCUSSION

On the basis of the above findings one can tentatively conclude that the protection of the HMM/LMM hinge is due to the highly ordered close 'packing' of myosin-containing thick filaments, and actin- and regulatory protein-containing thin filaments in the myofibrillar structure. This close packing does not prevent trypsin entering into the myofibril and reacting with myosin, as demonstrated by the rapid cleavage at the 25 kDa site from the NH_2 terminus of myosin which also takes place in myofibrils. It seems that, while the packing of proteins in the myofibrillar structure does not influence the proteolysis of the myosin head, it has a profound effect on the cleavage of the rod which becomes less accessible due to its location in the backbone of the thick filament. The LMM segment of the myosin rod is permanently located in the filament backbone; however, the S-2 segment which goes from the HMM/LMM hinge to the head (fig.1) can be attached or detached from the backbone depending on the ambient conditions such as pH and temperature [18–20]. According to Ueno and Harrington [21] the HMM/LMM hinge region is exposed to proteases only when it is detached from the filament backbone. Under the conditions of our experiments (rigor, pH 7.0 and 25°C) the S-2 segment seems therefore to be closely attached to the backbone of the thick filament in the intact myofibrils.

In conclusion, the present results bear on the dependence of the chemical properties of a macromolecule on the supramolecular structure and this dependence should be taken into account when in vitro results are extrapolated to explain phenomena taking place in highly organized biological structures.

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