Local Conformational Change of Myosin Detected by Tryptic Digestibility

II. Effect of Magnesium Ions on the Fragmentation Pattern of Heavy Meromyosin by Trypsin

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Received for publication, April 30, 1974

In order to detect the local conformational changes in the heavy meromyosin (HMM) molecule induced by magnesium ions, which have been suggested by the decrease in the ATPase [EC 3.6.1.3] activity of HMM caused by tryptic digestion, HMM with minimum cleavage of the intramolecular peptide bond, prepared by tryptic proteolysis at pH 6.4, was digested with trypsin [EC 3.4.21.4] at pH 7.6 in the presence or absence of Mg ions. The following results were obtained.

1. The gel filtration pattern indicated that HMM subfragment-1 (HMM S-1) was obtained from HMM by tryptic digestion in the absence of Mg ions, but not in their presence. These results were not affected by the addition of substrate or analogues such as ATP, ITP, ADP, and PPi, or by the addition of actin.

2. SDS-polyacrylamide gel electrophoresis revealed that on tryptic digestion without the divalent cation, the large polypeptide chains contained in HMM were promptly degraded to form smaller peptides, while the presence of Mg ions almost completely prevented the formation of the peptide of approximately $4.2 \times 10^{-4}$ daltons.

3. The identification of the small peptides suggests the presence of flexible regions susceptible to trypsin, which are made rigid by Mg ions, in HMM S-1 and the linking part between HMM S-1 and HMM S-2.

It is well-known that Mg is essential for the superprecipitation and ATPase [EC 3.6.1.3] activity of actomyosin, the primary mechano-

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Abbreviations: HMM, heavy meromyosin; HMM S-1, HMM subfragment-1; HMM S-2, HMM subfragment-2; SDS, sodium dodecyl sulfate; LMM, light meromyosin.
(HMM) digested in the presence and absence of divalent cations, Biró et al. (2, 3) and Oppenheimer et al. (4) obtained HMM S-1 directly by tryptic digestion of myosin at a low ionic strength in the presence of \(10^{-2}\) M \(\text{Ca}^{2+}\). Recently it has been reported by Bálint et al. (5, 6) that HMM S-2 is produced by tryptic digestion of myosin in the presence of EDTA, while the formation of HMM S-2 was suppressed by divalent cations, and that in the presence of EDTA, HMM was also split into HMM S-1 and HMM S-2.

In this report, the effect of divalent ions, especially \(\text{Mg}\), on the conformation of the HMM molecule was further studied in detail by gel filtration and SDS-disc electrophoresis, and results were obtained suggesting that not only the fragmentation of HMM but also the peptide bonds of the HMM molecule susceptible to trypsin are very much affected by \(\text{Mg}\) ions.

**MATERIALS AND METHODS**

**Preparation of Myosin A, HMM, and G-actin**—Preparations of myosin A and HMM were performed as described previously (1), unless otherwise noted. G-Actin preparation was obtained from acetone-dried powder of rabbit skeletal muscle by the method of Mommaerts (7).

**Tryptic Digestion Procedure**—Tryptic digestion of HMM was carried out as described previously (1).

**Gel Filtration**—The tryptic digestion mixture was applied to a column (2.8×40 or 1.8×33 cm) of Sephadex G-200 previously equilibrated at 5° with 0.05 M KCl, 1 mM EDTA, and 0.01 M Tris-HCl, pH 7.5. The same buffer was used as the eluant at a rate of 20–30 ml per hr and the protein concentration was followed by determining the absorbance at 280 nm, using a Hitachi type 034 spectrophotometer.

**Disc-Gel Electrophoresis**—Runs were made in 10% or 7.5% polyacrylamide gels in the presence of sodium dodecyl sulfate according to the method of Weber and Osborn (8) with a slight modification. The proteins (5 mg/ml) were incubated at 55° for 2 hr in 0.01 M sodium phosphate buffer, pH 7.2 containing 1% SDS, 25% glycerol, and 1% \(\beta\)-mercaptoethanol. Bromphenol blue was used as a tracking dye. After incubation, 20 \(\mu\)l of the protein solution was applied on the gels, using 1 : 37, w/w cross-linker. Electrophoresis was performed at a constant current of 8 mA per gel in 0.1 M sodium phosphate buffer (pH 7.2) containing 0.1% SDS at room temperature. Under these conditions the marker dye moved about 8–10 cm in approximately 8 hr. After electrophoresis, the gels were stained with 0.5% amide black 10B in 20% acetic acid solution at 50° for 2 hr. For destaining, 7% acetic acid solution was used. For molecular weight determination, a mixture of marker proteins was run in parallel.

**Reagents**—Trypsin [EC 3.4.21.4], soybean trypsin inhibitor and nucleotides were commercial products.

**RESULTS**

**Fragmentation of HMM by Tryptic Digestion**—Since the action of the nucleotide on the tryptic inactivation of ATPase is markedly affected by \(\text{Mg}\) ions, as reported previously (1), the fractionation of digests of HMM treated with trypsin in the presence and absence of \(\text{Mg}^{2+}\) was done by gel filtration. Figure 1-A represents a typical elution profile of HMM digested with trypsin for 5 min in the absence of divalent ions (i.e., in the presence of EDTA), showing three major peaks. This elution pattern is in good accord with that reported by other investigators (6, 9). Peak I, invariably appearing in the void volume, had the same ATPase activity as the peak excluded on the gel filtration of undigested HMM, as shown in Fig. 1-C, suggesting that peak I consists mainly of undigested HMM. Peak II, as indicated in Fig. 1-C, seems to be mostly the trypsin-trypsin inhibitor complex. The other retarded peaks appear to be small peptide fragments released from HMM. The elution diagram ob-

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Fig. 1. Column chromatography of trypsin-digested HMM on Sephadex G-200 in the presence or absence of Mg\(^{2+}\). HMM (10 mg/ml) was digested with trypsin (weight ratio of HMM to trypsin = 10 : 1) for 5 min in 0.5 M KCl, 0.1 M Tris-HCl buffer, pH 7.6, at 20°. The digestion was stopped by adding trypsin inhibitor (twice the amount of trypsin). The total volume (10 mg/1.2 ml) of the digested HMM solution was applied to a column (1.8 x 33 cm) of Sephadex G-200 equilibrated at 5° with 0.01 M Tris-HCl buffer, pH 7.5, containing 1 mM EDTA and 0.05 M KCl. A, HMM digested in the presence of 2 mM EDTA; B, HMM digested in the presence of 1 mM MgCl\(_2\); C, undigested HMM with trypsin-trypsin inhibitor complex. ATPase activity (\(\times\)) was determined in 0.1 M KCl at 37°, pH 7.6 (20 mM histidine buffer), in the presence of 5 mM CaCl\(_2\) and 1 mM ATP.

Fig. 2. SDS-polyacrylamide gel electrophoretic pattern of digested HMM. Electrophoresis was done using 10% gels for 8 hr at 8 mA/tube. Electrophoreograms were obtained by scanning gels stained with amide black 10B at 600 nm. A, undigested HMM with trypsin-trypsin inhibitor complex; B, HMM digested for 5 min in the presence of 2 mM EDTA; C, HMM digested for 5 min in the presence of 1 mM MgCl\(_2\). HMM prepared by the method of Young \textit{et al.} was used. For other details, see the text.

Subsequently, the effect of substrate and its analogues on the fragmentation of HMM by trypsin was studied and following results were obtained; the elution patterns of HMM's digested in the presence of EDTA-ATP, EDTA-ADP, EDTA-ITP, and EDTA-PP\(_i\) were almost the same as that in the presence of EDTA alone (Fig. 1-A), while in the presence of Mg\(^{2+}\)-ATP, Mg\(^{2+}\)-ADP, Mg\(^{2+}\)-ITP, and Mg\(^{2+}\)-PP\(_i\), fractionation patterns of the tryptic digest of HMM were almost the same as that of the sample digested in the presence of Mg\(^{2+}\) alone (Fig. 1-B). It is concluded from these data that the fragmentation of the HMM molecule by trypsin is not affected by nucleotides but is markedly affected by divalent cations alone, although the tryptic inactivation of ATPase activity is profoundly affected by nucleotides (1).

Furthermore, the effects of KCl concentration, LMM, which has a great influence on the solubility of myosin, and actin on the fragmentation of HMM by trypsin were investigated. As shown in Table I, KCl concentration had no effect on the tryptic digestion of the contractile protein. Neither LMM nor actin were effective. Therefore, the peptide bonds of the HMM molecule susceptible to trypsin seem to be affected only by divalent
cations under the conditions used. The minimum effective concentration of Mg\(^{2+}\) was 10\(^{-5}\) M. Although Ca ions can be substituted for Mg ions, peak I (Ca\(^{2+}\)) frequently has a small shoulder (not shown here), suggesting that the action of Ca ions is a little different from that of Mg ions on the fine conformation of the molecule. Surprisingly, on digestion of myosin with trypsin under the same conditions as reported by Gráf et al. (3), peak II always emerged irrespective of KCl concentration and the presence of divalent cations in the digestion mixture. It seems that borate has some special action on the tryptic digestibility of myosin, although its nature is not clear.

**Cleavage of Polypeptide Chain of HMM by Trypsin**—In order to detect the degree of splitting of peptide bonds of the HMM molecule by trypsin, SDS-disc electrophoretic patterns of HMM digested in the absence and presence of Mg\(^{2+}\) were investigated, as shown in Fig. 2, where the molecular weight of each band was estimated from a standard sample run under identical conditions. As seen in Fig. 2-A, undigested HMM prepared by the method of Young et al. (9) showed one major band (molecular weight>10\(^{10}\) daltons; referred to as P0), including a few minor components. Remarkable differences between electrophoreograms of digested HMM's in the absence and presence of Mg\(^{2+}\) were observed with a few polypeptide chains, especially P4 (5.5\(\times\)10\(^4\)) and P6 (4.2–4.5\(\times\)10\(^4\)); in the presence of Mg\(^{2+}\) the production of P2 and P4 was considerably suppressed, while P6 was scarcely observed.

Figure 3 shows the electrophoretic pattern of peak II (HMM S-1), isolated by gel filtration from HMM digested in the absence of divalent cations. Polypeptide chains, P4 and P6 (2.6\(\times\)10\(^4\)), were observed as main components.

**Binding of Tryptic Digest to Actin**—The supernatant after digested HMM had been sedimented together with F-actin was subjected

### TABLE I. Effects of divalent cations, KCl concentration and buffer on the tryptic digestibility of HMM, myosin, and actomyosin. Tryptic digestion of HMM was carried out in 0.5 and 0.02 M KCl. Myosin digested for 5 min in 0.5 M KCl was dialyzed against 0.02 M KCl and centrifuged. The supernatant was used for chromatography. Synthetic actomyosin (actin:myosin=1.45:1 by weight) was digested in 0.083 M KCl (*) at 20° for 5 min, the weight ratio of trypsin to protein being 1:16. Digested actomyosin was centrifuged at 105,000 \(\times\) g for 2 hr in the presence of 10 mM PPi and 5 mM MgCl\(_2\) at pH 7. The supernatant was used for chromatography. Tryptic digestion of myosin for 20 min in 0.02 M borate buffer (pH 8.3) at 20° was performed in the presence of 10 mM EDTA, Mg\(^{2+}\) or Ca\(^{2+}\) at 0.5 and 0.02 M KCl. The weight ratio of trypsin to myosin was 1:100. After dialysis of the myosin digest against 0.05 M KCl-0.01 M Tris-histidine buffer (pH 7.6), the supernatant was subjected to chromatography. For other details, see the text.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Buffer used for digestion</th>
<th>Divalent cation or chelator added</th>
<th>Appearance of HMM S-1 (0.5 M KCl)</th>
<th>Appearance of HMM S-1 (0.02 M KCl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heavy meromyosin</td>
<td>Tris-HCl (pH 7.6)</td>
<td>2 mM EDTA</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1 mM Mg(^{2+})</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Myosin</td>
<td>Tris-HCl (pH 7.6)</td>
<td>2 mM EDTA</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1 mM Mg(^{2+})</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Actomyosin</td>
<td>Tris-HCl (pH 7.6)</td>
<td>2 mM EDTA</td>
<td>+*</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1 mM Mg(^{2+})</td>
<td>-*</td>
<td></td>
</tr>
<tr>
<td>Myosin</td>
<td>Borate (pH 8.3)</td>
<td>10 mM EDTA</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10 mM Mg(^{2+})</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10 mM Ca(^{2+})</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

The + signs in the table represent the appearance of peak II, corresponding to HMM S-1, in the gel filtration pattern of digested contractile protein.
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Fig. 3. Densitogram of HMM S-1 isolated by gel filtration. HMM digested in the presence of EDTA was applied to a Sephadex G-200 column under the same conditions as in Fig. 1. Peak II was collected, condensed, and subjected to electrophoresis. For other details, see the legend to Fig. 1 and the text.

Fig. 4. Electrophoreograms of the supernatants after the tryptic digest of HMM had been centrifuged together with actin. To the tryptic digest of HMM, actin was added, the weight ratio of actin to HMM being 1:2. After centrifugation of the mixture at 105,000 × g for 3 hr, the supernatant was subjected to electrophoresis. A, the tryptic digestion of HMM in the presence of EDTA. B, the tryptic digestion in the presence of MgCl₂. The numbers indicate the digestion time (min).

to electrophoresis. With the tryptic digest treated in the absence of Mg²⁺, three peptide chains including P₆ were observed in the electrophoretic pattern, as shown in Fig. 4. Control experiments indicated that two polypeptide chains other than P₆ were a denatured G-actin and tropomyosin. With the degraded sample in the presence of Mg²⁺, however, P₆ was not found at all in the supernatant.

DISCUSSION

As previously reported (1), the rates of hydrolysis of p-toluenesulfonyl-L-arginine methyl ester (TAME) and casein with trypsin increased on addition of divalent cations but were not affected at all by addition of the nucleotide. Based on the amounts of TCA-soluble non-protein nitrogen in the digestes, however, it was concluded that the hydrolysis of HMM by trypsin was not accelerated but rather was somewhat suppressed on addition of the divalent cations, while nucleotides were hardly effective on the tryptic hydrolysis. On the other hand, the inactivation of ATPase activity by tryptic digestion was profoundly affected by divalent cations and nucleotides. Thus, Mg ions seem to influence the structure of myosin and HMM in two different ways; one affects the active site of myosin ATPase and ATP is very effective here, as described previously (1), suggesting the need for Mg ions for the binding of ATP to the active site, as a result of which the conformation of the active site seems to be changed. The other area affected is a region other than the active site of myosin which is not affected by ATP or its analogues, as shown in this report. Recently studies on direct preparations of HMM S-1 from myosin or myofibril have been reported (2-6, 10-15). Among them, the studies by Biró et al. (2, 3) and by Bálint et al. (5, 6) are very interesting. Their early reports (2, 3) indicated that the tryptic digestion of myosin at low ionic strength in the presence of 10⁻² M Ca²⁺ directly produced an HMM S-1 like protein without cleavage of myosin into heavy and light meromyosins. Their latest reports (5, 6), however, have shown that the degradation of HMM to HMM S-1 and HMM S-2 is suppressed by alkaline earth metals and they have suggested that the discrepancy between their latest results and those previously obtained may be due to a decrease in resistance to proteolysis on aging. On the other hand, our data (Table I) showed that the tryptic digestion of a freshly prepared myosin in borate buffer produced...
an HMM S-1 like protein, whether divalent cations were present or not in the digestion mixture and that the fragmentation of myosin by trypsin did not produce HMM S-1 at all in the presence of Mg ions. In the experiments newly reported by Bálint et al., Tris buffer was used instead of borate buffer. Therefore, borate, a kind of chelator, appears to counteract the effect of divalent cations.

The cleavage of HMM to HMM S-1 by tryptic digestion is markedly affected by Mg ions, its effect being apparent at $10^{-5}$ M and saturated at $10^{-4}$ M. It is suggested, therefore, that one mole of Mg ions per mole of HMM can produce conformational changes of the HMM molecule, since the digestion mixture contains $3 \times 10^{-5}$ M HMM (3.2 $\times 10^{3}$ daltons).

The data obtained lead us to the following conclusion: A region sensitive to proteolytic attack seems to be present close to the globular portion of HMM, i.e., between HMM S-1 and HMM S-2 and the conformation of the region is not affected by the type of monovalent ion, ionic strength, nucleotides, or actin but only by divalent ions.

Among the bands seen on SDS-gel electrophoresis, the appearance of bands with molecular weights of $5.5 \times P_{4}$ and $4.2-4.5 \times P_{5}$ daltons was markedly affected by Mg ions, as seen in Fig. 2. $P_{4}$ and $P_{5}$ obviously appeared within 2–3 min on tryptic digestion in the absence of Mg$^{2+}$ while in the presence of Mg$^{2+}$, the appearance of $P_{4}$ was considerably suppressed, that of $P_{5}$ being almost completely suppressed. $P_{4}$ and $P_{5}$ (2.6 $\times 10^{-4}$ daltons) are implicit in HMM S-1 preparations, as reported by Hayashi (16). This indicates, therefore, that Mg ions inhibit not only the degradation of HMM into HMM S-1 and HMM S-2, but also the cleavage of a peptide bond present in HMM S-1 which results in the formation of the smaller components, $P_{4}$ and $P_{5}$. When HMM digested in the absence of Mg ions was centrifuged with actin, the component $P_{5}$ was absent in the sediment but present in the supernatant (Fig. 4), suggesting the possibility that this component may represent a single chain of the double-stranded subunits of HMM S-2, if we take into consideration the molecular weight of HMM S-2 reported by Bálint et al. (6).

The results obtained so far suggest that Mg ions are essential for muscle contraction, not only through the fixation of the substrate to the active site of myosin, but also through the maintenance of the head and neck regions of the myosin molecule in a definite tertiary structure.

We wish to express our deep gratitude to Prof. T. Sekine for his valuable advice and continuous encouragement during this work.

REFERENCES