Thermal stability of myosin subfragment-1 decreases upon tryptic digestion in the presence of nucleotides

Katalin Pintér*, Renne Chen Lu and László Szilágyi*

*Department of Biochemistry, Eötvös Loránd University, Budapest, Puskin u. 3, H-1088, Hungary and Boston Biomedical Research Institute, 20 Staniford St., Boston, MA 02114, USA

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Myosin subfragment-1 (S-1), digested with trypsin in the presence of ATP, rapidly loses its ATPase activity upon mild heat treatment even if ATP or ADP is present. The heat-treated molecule is very sensitive to further tryptic digestion. Undigested S-1 and S-1 digested in the absence of ATP are protected by nucleotides. The loss of the protective effect of nucleotides correlates with the tryptic splitting of the 25 kDa amino-terminal fragment between Arg 23 and Ile 24.

Myosin subfragment-1 *Tryptic digestion* *Thermal stability*

1. INTRODUCTION

To explain the molecular mechanism of energy transformation in contracting muscle, it has been proposed that the active machinery of the contraction lies in the myosin heads which contain two spatially separate but interacting binding sites for nucleotides and actin [1]. A great deal of experimental evidence supporting this attractive hypothesis has come from studies using limited proteolysis. Trypsin and several other proteases cut the heavy chain of myosin S-1 at two places generating 3 large fragments of about 25, 50 and 20 kDa [2–6]. The protease-sensitive 'connector' regions are presumably loose loops on the surface of the protein, and the large fragments are believed to form structural domains in the myosin head [5,7]. Recently, two papers were published about the effect of thermal denaturation on the proteolytic fragmentation pattern of the heavy chain in myosin heads. Tryptic digestion of HMM at 37°C results in complete decomposition of the 3 fragments with a simultaneous decrease in ATPase activity [8]. This observation is in accordance with the well-known fact that myosin readily denatures at 35°C [9,10]. It was also reported [8] that the presence of Mg-ADP during heat treatment and digestion helped to conserve the original fragmentation pattern. Setton and Muhlrad [11] reported that tryptic digestion of S-1 having been incubated at 35°C for 1–2 h led to the complete loss of the 50 kDa fragment, while the 25 and 20 kDa fragments seemed to be stable. If Mg-nucleotides were present during heat treatment and digestion, they protected the 50 kDa fragment.

To show whether the fragmentation of S-1 heavy chain has any effect on the thermal stability of the molecule, we studied 3 different S-1 species, namely chymotryptic S-1 with intact heavy chain (95 kDa S-1), S-1 digested with trypsin in the absence of nucleotides (25 kDa S-1) and S-1 digested in the presence of Mg-ATP (21 kDa S-1). Here, we report that Mg-ATP protects both 95 kDa S-1 and 25 kDa S-1 during heat treatment, but it is ineffective in the case of 21 kDa S-1.

* To whom correspondence should be addressed

Abbreviations: S-1, myosin subfragment-1; 95 kDa S-1, S-1 with intact heavy chain; 25 kDa S-1, digested S-1 consisting of 25, 50 and 20 kDa fragments; 21 kDa S-1, digested S-1 consisting of 21, 50/47 and 20 kDa fragments

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2. MATERIALS AND METHODS

Trypsin, α-chymotrypsin and soybean trypsin inhibitor were obtained from Sigma. ATP and ADP were purchased from Reanal (Budapest).

Chymotryptic S-1 was prepared as in [12]. The concentration of S-1 was estimated by UV absorption employing $E^{1\%}_{280nm} = 8.0$. 25 kDa S-1 was obtained by tryptic digestion using a 1:100 (w/w) ratio of trypsin and S-1 at 25°C for 60 min. To prepare 21 kDa S-1, 5 mM ATP or ADP was added, and the trypsin/S-1 ratio was 1:50 (w/w). The concentration of S-1 was 4–6 mg/ml in both cases, and the medium contained 50 mM KCl, 50 mM Tris-HCl, pH 8, and 5 mM $MgCl_2$. Digestion was terminated by adding soybean trypsin inhibitor at a 1.5:1 (w/w) ratio to trypsin. Digested S-1 was precipitated with 65% ammonium sulfate in the presence of 5 mM EDTA (final concentration). Precipitates were dissolved in a solution containing 50 mM KCl and 50 mM Tris-HCl, pH 8. Protein concentration was measured by the Coomassie dye binding method [13] using undigested S-1 as standard.

Samples containing 4 mg/ml S-1 (intact or digested), 50 mM KCl, 50 mM Tris-HCl, pH 8, and either 2.5 mM $MgCl_2$ alone or both 2.5 mM $MgCl_2$ and 5 mM ATP or ADP, were incubated at 35°C, and aliquots were taken for ATPase measurements at several times. Following heat treatment, the samples were digested with trypsin at 25°C using a 1:50 (w/w) ratio for 5–30 min. The required amount of trypsin was calculated by taking into account the trypsin inhibitor content of the samples.

SDS gel electrophoresis was performed on slab gels as in [14] using 14% polyacrylamide as separating gel. Gels were stained with Coomassie brilliant blue R250. The relative intensities of the stained bands were assessed by scanning the gels at 560 nm using the linear transport attachment of a Beckman 26 spectrophotometer.

K^+-activated ATPase activities were determined by measuring the released P_i [15]. The tests were carried out on 1-ml volumes incubated at 20°C for 1 min, and containing 40 μg S-1, 1 M KCl, 5 mM EDTA, 50 mM Tris-HCl, pH 8, and 2 mM ATP.

The 21 kDa fragment was isolated by adapting the method used for isolating the 25 kDa fragment in [7]. Edman degradations were carried out in a Beckman 890C sequencer using program 030176. 2 mg polybrene was routinely added to the sample before application to the cup [16]. Phenylthiohydantoins were identified by HPLC as in [17].

3. RESULTS AND DISCUSSION

In the first set of experiments we incubated each S-1 species at 35°C with and without ATP or ADP, and the inactivation was monitored by measuring the K^+-activated ATPase activities of aliquots (fig.1). In the absence of nucleotides the inactivation of 21 kDa S-1 was very rapid, about 80% of the activity being lost within 20 min. The other two S-1 species were also sensitive to heat treatment, although their inactivation was somewhat slower. There was a greater difference when nucleotides were present during the heat treatment. ATP or ADP prevented the loss of ATPase activity of 95 kDa S-1 and 25 kDa S-1, but were ineffective in the case of 21 kDa S-1. The inactivation of this S-1 was very rapid irrespective of the presence or absence of nucleotides.

It should be mentioned that fragmentation itself

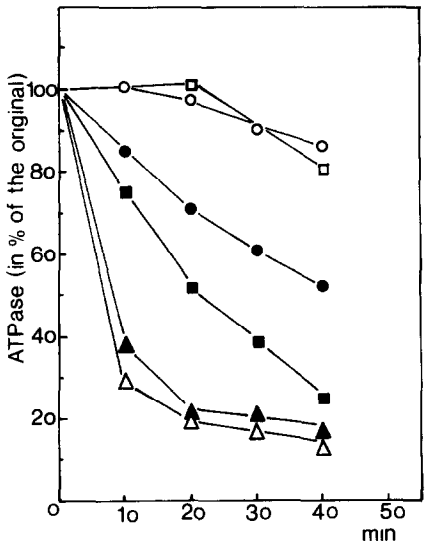

Fig.1. Time course of inactivation of S-1 and S-1 derivatives in the absence (closed symbols) and presence (open symbols) of 5 mM ADP at 35°C. (●,○) 95 kDa S-1, (■, □) 25 kDa S-1, (▲, △) 21 kDa S-1. The ATPase activity of each S-1 was about 8 s^{-1} before thermal treatment.

does not alter the ATPase activity of S-1. K^+-activated ATPase activity of each S-1 species, including 21 kDa S-1, was about 8 s^{-1}.

When the heat-treated S-1 species were subjected to tryptic digestion, 95 kDa S-1 and 25 kDa S-1 showed the regular fragmentation pattern if nucleotides were present during both thermal treatment and subsequent tryptic digestion. When heat treatment of these two S-1s was done without nucleotides, degradation of the fragments by trypsin was very rapid, and there was a considerable loss of 50 kDa fragment in both cases (fig.2A,B). This observation confirms the results in [11].

Tryptic digestion of heat-treated 21 kDa S-1 resulted in a rapid decrease in intensities of the 50, 47, 21 and 20 kDa bands irrespective of the presence or absence of nucleotides. At the same time, however, a strong band of about 25 kDa appeared. This is evidently a degradation product of the 50 kDa fragment, because 21 kDa S-1 contains only an insignificant amount of 25 kDa fragment. The possibility of such a transformation in heat-denatured S-1 was also suspected in [11]. The results described above indicate that tryptic digestion of S-1 in the presence of nucleotides yields a derivative which is extremely sensitive to thermal denaturation, and its native state cannot be protected by nucleotides. Our results also show that different parts of the structure of the 50 kDa fragment are not equally sensitive to thermal denaturation.

The presence of nucleotides during tryptic digestion induces two additional cleavages in the heavy chain of S-1 leading to a faster 25 kDa ⟶ 21 kDa and a slower 50 kDa ⟶ 47 kDa transformation [8,18,19]. To determine which transformation is responsible for destabilizing the molecule, the following experiment was performed. S-1 was digested in the presence of 5 mM Mg-ADP for 90 min and aliquots taken at several times. Digestion was stopped by addition of soybean trypsin inhibitor. The ATPase activity of the samples corresponding to different degrees of digestion was measured before heat treatment and after 40 min incubation at 35°C. Activities remaining after thermal treatment were expressed in percentage of the activities measured before heat treatment (fig.3A). An equal volume of each sample was also saved for SDS gel electrophoresis. The electrophoretogram of the samples (fig.3B) was scanned and we expressed the relative amount of 50 and 25 kDa fragments as a percentage of 50 + 47 kDa and 25 + 21 kDa, respectively (fig.3A). We found that the temperature-induced inactivation paralleled the 25 kDa ⟶ 21 kDa transformation as seen in fig.3A. At the same time, there is also a 50 kDa ⟶ 47 kDa transformation, but this conversion is not complete even after pro-

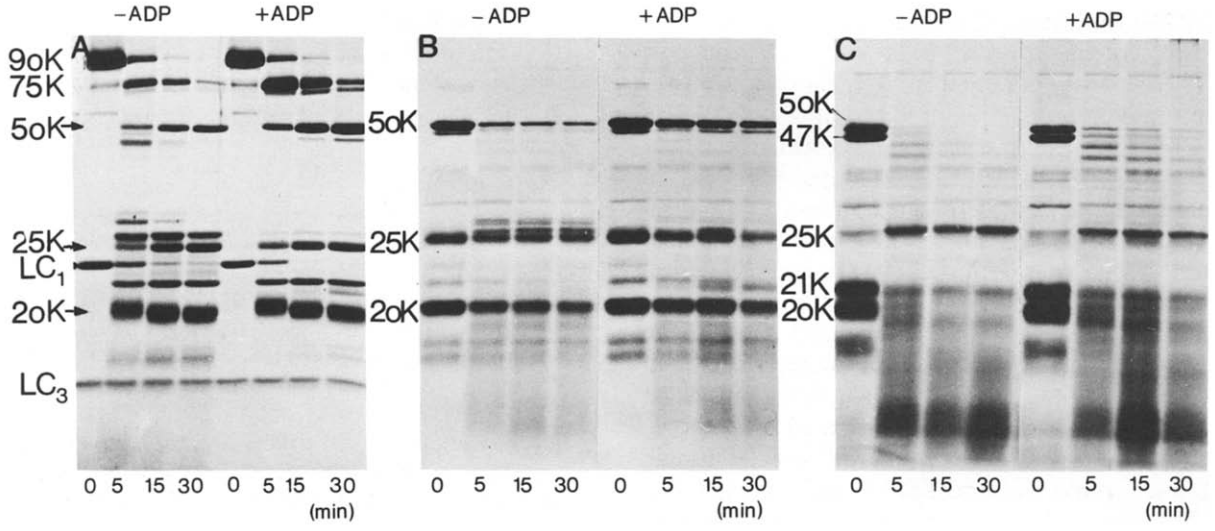

Fig.2. Tryptic digestion of the 3 S-1 species after heat treatment. The samples were incubated with and without 5 mM ATP at 35°C for 2 h and were then digested with trypsin at 25°C. (A) 95 kDa S-1, (B) 25 kDa S-1, (C) 21 kDa S-1.

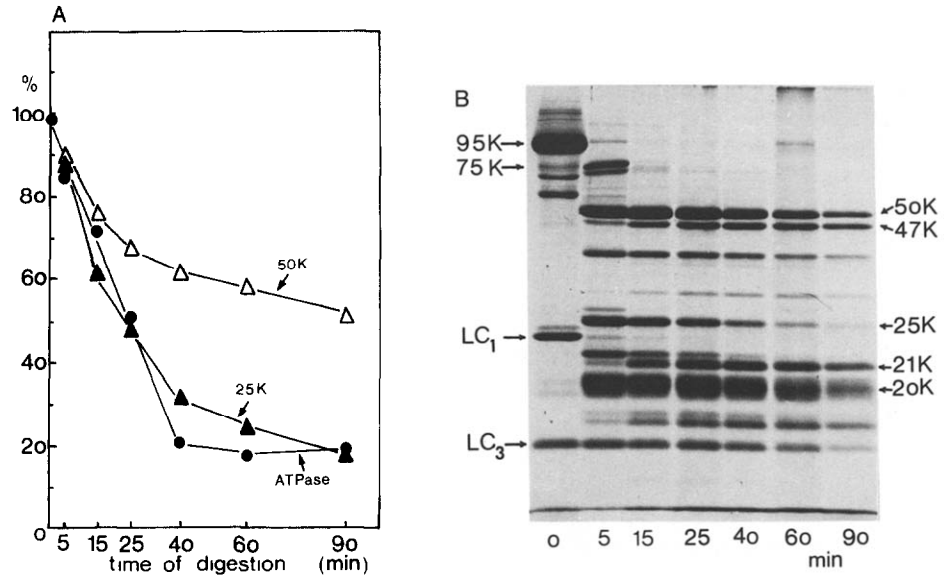

Fig.3. Influence of tryptic digestion on the thermal stability of S-1. 95 kDa S-1 was digested in the presence of 5 mM ADP at 25°C and aliquots were taken for measurement of ATPase activity before and after heat treatment and for SDS gel electrophoresis. (A) ATPase activities expressed as a percentage of the activities measured before the incubation at 35°C for 40 min (●). Samples contained 5 mM ADP during thermal treatment. The relative amounts of 50 kDa (△) and 25 kDa (▲) fragments are expressed as a percentage of 50 kDa + 47 kDa and 25 kDa + 21 kDa fragments, respectively. (B) SDS gel electrophoretogram used for densitometry.

longed proteolysis. We suppose, therefore, that the loss of the protective effect of nucleotides is due to the splitting in the 25 kDa fragment.

To localize the position of the nucleotide-induced tryptic splitting in the primary sequence of the 25 kDa fragment, we isolated the 21 kDa fragment and determined its amino-terminal sequence. We found that the 21 kDa fragment does not contain the acetylated amino terminus of myosin heavy chain, although it was localized in the 25 kDa fragment [20]. The amino-terminal sequence of the 21 kDa fragment is Ile-Glu-Ala-Gln-Asn-Lys-Pro-Phe-Asp-Ala. This sequence is identical with that starting at Ile 24 in the amino-terminal fragment of S-1 as reported in [21]. This indicates that the nucleotide-induced cleavage occurs between Arg 23 and Ile 24. Our result confirms the amino-terminal sequence of the 21 kDa fragment reported in [19].

In summary, we conclude that the additional tryptic splittings induced by the presence of nucleotides, most probably the cleavage between Arg 23 and Ile 24, greatly reduces the stability of the protein even in the presence of nucleotides. Circumstantial evidence shows that at least the ATP-binding ability of 21 kDa S-1 is not significantly impaired since the K^+- and Mg^{2+}-activated ATPase activities of this S-1 are nearly identical to those of 95 kDa S-1 and 25 kDa S-1. Consequently, the loss of the protective effect of ATP in 21 kDa S-1 reflects such alterations which prevent the propagation of structural changes from the active site to the whole of the molecule.

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