Vol. 89, No. 3, 1979 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
August 13, 1979 Pages 925-932

THE LIMITED TRYPTIC CLEAVAGE OF CHYMOTRYPTIC S-1 : AN APPROACH TO THE CHARACTERIZATION OF THE ACTIN SITE IN MYOSIN HEADS

D.MORNET, P.PANTEL, E.AUDEMARD and R.KASSAB
Centre de Recherches de Biochimie Macromoléculaire du CNRS, Route de Mende, BP 5051, 34033 Montpellier Cedex
(France)

Received May 11,1979

SUMMARY : Limited tryptic proteolysis of S-1 (A_1+A_2) or S-1 (A_1) and S-1 (A_2) converts the heavy chain into 3 fragments of Mr = 27K-50K-20K. As a result the actin-stimulated ATPase activity of the fragmented heads is lost. When the digestion is performed using the complex F-actin-S-1, this ATPase activity is completely preserved and the heavy chain is split into only 2 fragments of Mr = 27K-70K. The specific protection by F-actin of the -COOH terminal region of the heavy chain at the joint 50K-20K against tryptic cleavage and loss of activity suggests that this part of the head can be involved in actin binding site and/or Mg^{2+} ATP hydrolysis by the acto-S-1 complex.

INTRODUCTION

The heavy chain of papain HMM-S1 is specifically split by trypsin into 3 fragments of Mr = 25K, 50K, 20K (1). Starting with the idea that these three fragments may constitute in the native enzyme three discrete domains, each having a specific biological function, we decided to study the effects of tryptic fragmentation of chymotryptic S-1 on its enzymic properties. Because the interaction of actin with myosin heads plays a key role in muscle contraction, we decided to investigate carefully actin binding and Mg^{2+}ATPase activation in the course of the proteolysis as well as the susceptibility of the S-1 molecule to trypsin when it is complexed to F-actin. The results led us to discover that the structural integrity of the COOH-terminal region of the heavy chain at the joint 50K-20K is essential for the expression of the actin stimulated ATPase of S-1; This particular part of the head which lies close to the hinge domain of myosin appears to be involved in actin binding and Mg^{2+}ATP hydrolysis by the acto S-1 complex.

MATERIAL and METHODS

Rabbit squeletal muscle myosin is prepared according to Offer (2); S-1 is prepared by digestion of myosin filaments (3,4) with chymotrypsin (Worthington); it is purified on Sephacryl S-200 in 100 mM potassium bicarbonate buffer pH 8.0; pure isoenzymes S-1 (A_1) and S-1 (A_2) were separated by chromatography on DEAE-cellulose (Whatman DE-52) (3,4). Actin is prepared as described by Straub (5) and purified according to Spudich and Watt (6). Protein concentrations are estimated from A 280 nm = 5.5 cm^{-1} for myosin (7) 7.5 cm^{-1} for S-1 species (4) and 11.0 cm^{-1} for actin (8). Determination of K^+-EDTA-, Ca^{2+}, Mg^{2+}- and actin stimulated ATPase activities is performed as described (9) using an automated phosphate analyzer designed as described by Terasaki and Brooker (10).

Fragmentation of chymotryptic S-1 (2 mg/ml) with TPCK-trypsin (Worthington) is carried out at E:S = 1:100 (w/w) in 0.1 M potassium bicarbo-

0006-291X/79/150925-08$01.00/0

nate pH 8.0, 25°C during 30 minutes in the absence and presence of F-actin (molar ratio F-actin : S-1 = 2). At suitable time intervals, 25-50 µl aliquots are withdrawn and essayed for ATPase activities; in parallel another 50 µl aliquot is taken and added to an equal volume of boiling 2% SDS, 5% ß-mercaptoethanol solution; after 5 minutes at 100°C, samples were subjected to SDS-polyacrylamide gel electrophoresis. A high resolution slab gel electrophoresis system was used according to Laemmli (12) Studier (13) and O'Farrel(14); the acrylamide and bis-acrylamide concentrations in the gels are 15% and 0.4% respectively. A Joyce-Loebel densitometer is used for scanning dried gels; molecular weights were estimated using pure commercial marker protein as well as myosin light chains and rabbit myofibrillar proteins (15).

Association and dissociation reactions of the complexes of actin with native and trypsin modified S-1 were studied by turbidimetry (16).

RESULTS

Limited tryptic cleavage of chymotryptic S-1 in the absence and presence of F-actin : effects on ATPase activities and structure :

We have first studied the time course of the proteolysis by trypsin of S-1 (A_1+A_2) as well as S-1 (A_1) and S-1 (A_2) and we have analysed concomitantly its effects on the ATPase activities of the protein. Fig.1 shows that the heavy chain of Mr = 95K is converted into 3 main fragments according to a two steps process. The two components of Mr = 75K and 20K are first rapidly formed (t 1/2 about 1.5 min); the former is then cleaved at a lower rate into 2 fragments of Mr = 50K and 27K. Concerning the light chains only L1 is significantly modified; after 1-2 minutes reaction it is completely converted into a peptide of Mr = 23K and then into fragments of Mr = 19-17K; all these components are clearly deriving from L1 since they are present in the digest of pure S-1 (A_1) (Fig.1) and absent in the digest of pure S-1 (A_2). The time course of the fragmentation of the heavy chain of these two isolated S-1 species is identical to that of the parent prepara-

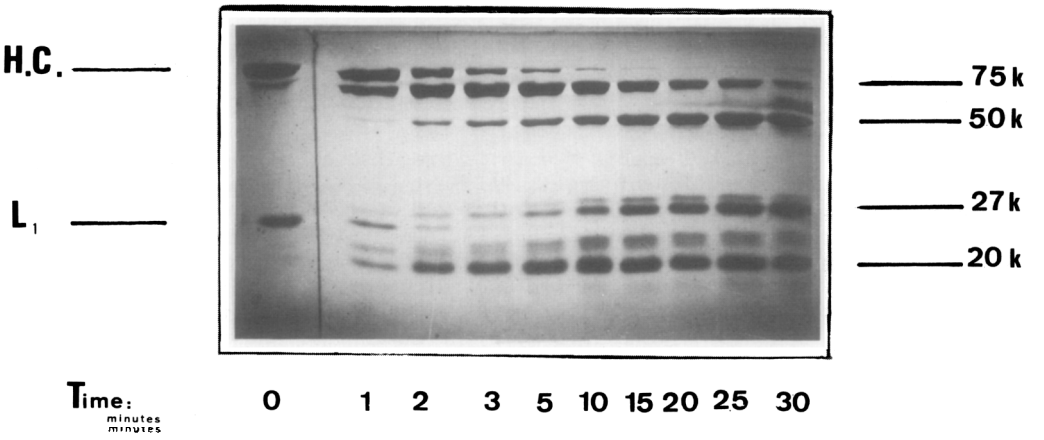


Figure 1.- Time course of limited cleavage of S-1 (A_1) by trypsin.

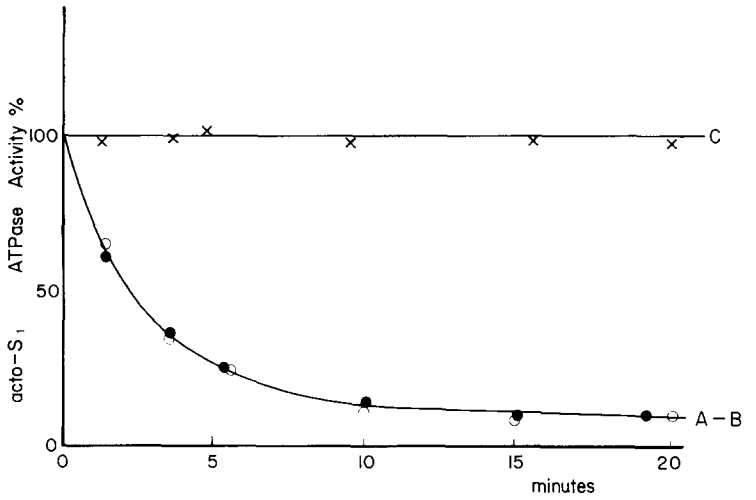


Figure 2.- Actin-activated ATPase of S-1 measured during trypsin digestion : A) proteolysis of S-1 (A_1+A_2)
B) proteolysis of S-1 (A_2)
C) proteolysis of the complex F-actin-S-1 (A_1+A_2)
100% activity = 3.2 µmoles Pi/min/mg S-1.

tion. Finally a study of the time course of changes in ATPase activities during proteolysis showed that the K^+-EDTA-, Ca^{2+}- and Mg^{2+}ATPases are unaltered; in contrast the actin-stimulated ATPase activity is rapidly lost and reaches after 10 minutes a constant value of 8-10% relative to that of the control with half-time of about 2 min. (Fig.2).

A critical comparison of the rate of this ATPase inactivation with the time course of the changes in the peptide pattern of the digest as measured by a quantitative densitometric scanning of the gels (Fig.3) strongly suggested that the inactivation is closely related to the first step of cleavage of the initial 95K heavy chain into the 75K + 20K fragments;the highest degree of inactivation is obtained concomitantly with the total disappearance of the parent heavy chain. The subsequent fragmentation of the 75K component cannot be involved being a relatively slow process neither can be the light chains since the degradation rate of L1 is too high and because, in particular, pure S-1 (A_2) loses similarly its actin-activated ATPase upon digestion (Fig.2) while L3 remains apparently unchanged on the gels. This assumption was strengthened by the results obtained when S-1 is treated by trypsin in the presence of F-actin. F-actin is known to be refractory to proteolysis (17) and therefore cannot compete with S-1 as substrate of trypsin. Fig.2 shows that under this experimental condition, the actin-activated ATPase is completely conserved and no alteration is observed in any other ATPase activity. Examination of the time course of the tryptic digestion of the acto-S-1

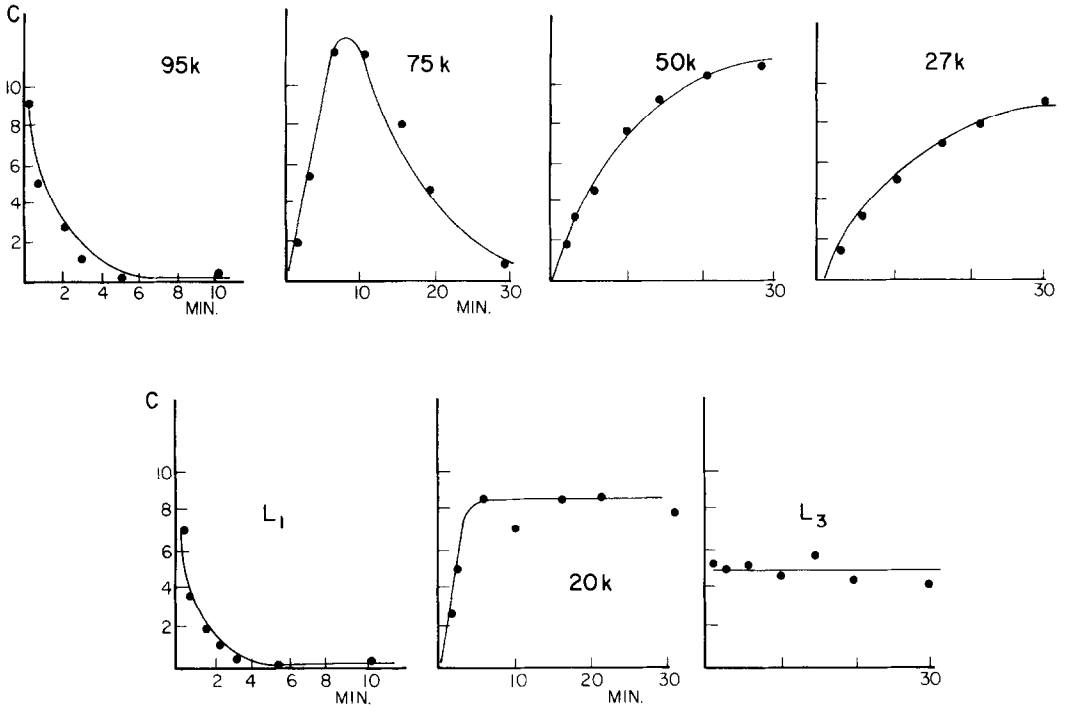


Figure 3.- Densitometric estimation of band intensities present in SDS gel slabs of S-1 during its fragmentation by trypsin. The band concentrations (C) are expressed with arbitrary values.

complex clearly shows a dramatic change in the fragmentation of S-1. The rapid appearance of the first two fragments 75K + 20K is completely abolished; instead the parent 95K heavy chain is slowly converted into two unique components of Mr = 70K + 27K (Fig.4). The light chain L1 is rapidly degraded as in the absence of F-actin with however accumulation of the corresponding 23K peptide. The rate of formation of 70K + 27K peptides compares well with that observed in absence of actin for the 50K + 27K peptides; thus the break at the junction of the 27K with 70K or 50K is unaffected by actin binding to S-1 and is not responsible for the actin-activated ATPase loss. The effect of actin is specific. S-1 digested in presence of Mg^{2+}ATP (5 mM) loses its actin-activated ATPase and gives a gel pattern not significantly different from that of S-1 alone. Two S-1 derivatives are isolated by gel filtration after 30 min. digestion of acto S-1 and S-1 alone; they contained respectively two and three heavy chain fragments and are refered to as "2-pieces" and "3-pieces" trypsin modified S-1. According to these results and those of Lu et al.(18) concerning the linear order of the 3 tryptic fragments along the S-1 heavy chain we can summarize the splitting action of trypsin on the

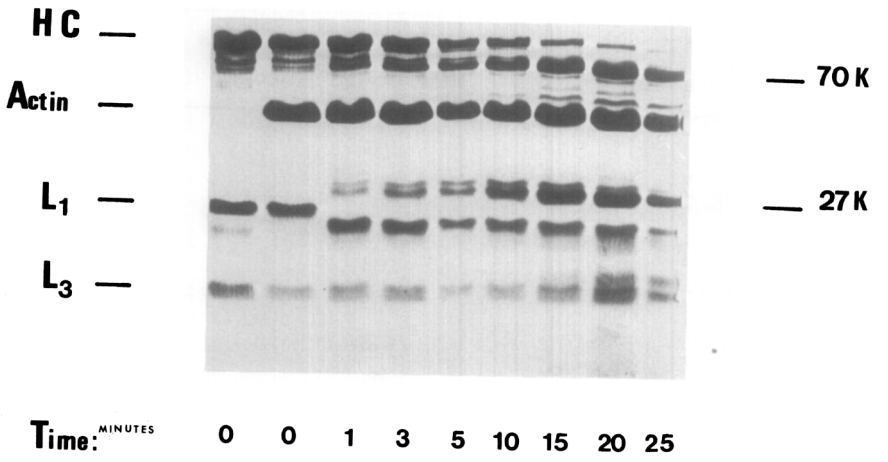
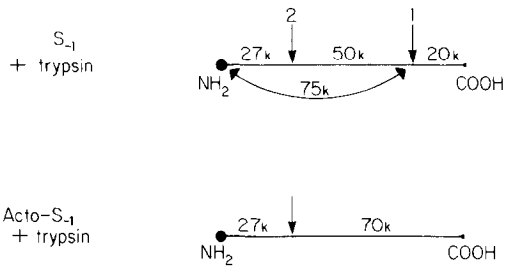


Figure 4.- Time course of limited cleavage by trypsin of the complex F-actin-S-1 (A_1 + A_2).

heavy chain of chymotryptic S-1 in absence and presence of F-actin as follow; the principal feature of this scheme is that actin protects only the COOH-terminal region of S-1 heavy chain :



Effect of limited trypsin proteolysis of S-1 on the association-dissociation properties of its complex with F-actin

Table I shows that during the course of fragmentation of the S-1 alone neither the association properties of the protein with F-actin nor the Mg^{2+} ATP induced dissociation of the complex are significantly altered althought the actin-activated ATPase is progressively lost. This result is in accord with the observed properties of the "3-pieces" trypsin modified S-1 to be isolated from the digest after combinaison with F-actin and dissociation of the complex by Mg^{2+} pyrophosphate. The cause of the ATPase loss appears to reside rather in the inability of actin to promote Pi release from the catalytic complex S-1-ADP.P when an internal bond cleavage has occured in the COOH-terminal region of the heavy chain.

Table I.- Turbidimetric measurements of acto-S-1 association-dissociation during treatment by trypsin.

DIGESTION TIME (minutes)	ACTO-S-1 ATPase	+ ACTIN	
		Without Mg^{2+} ATP	With Mg^{2+} ATP
0	100	105	70
1	70	110	70
2	45	110	80
3	35	105	80
5	20	105	78
10	10	110	75
15	8	105	75

S-1 0,1 mg in 10 mM Tris HCl, 100 mM KCl, 1 mM $MgCl_2$ buffer pH 8 (2 ml) are mixed with 0.2 mg of F-actin. Turbidimetry was measured at 400 nm 20°C in absence and presence of 5 mM $MgCl_2$ + 1.6 mM ATP. The values are arbitrary.

DISCUSSION

Our results on the fragmentation pattern of the heavy chain of chymotryptic S-1 by trypsin are in agreement with those described by Balint et al.(1) and Lu et al.(18) for papain HMM-S1 as well as by Cardinaud (19) for the same chymotryptic S-1. Further the high resolution slab gel system we used has indicated that the NH_2-terminal fragment has Mr = 27K and not 25K as reported by all these authors; it moves on gels more slowly than the 25K light chain L1(20). The great susceptibility of L1 and the stability of L3 to tryptic digestion were first observed by Hayashi (21) and then by Cardinaud (19) who showed the rapid conversion of L1 into a 23K component. Our work indicates that this derivative is further degraded into smaller peptides. A more precise insight into the fragmentation process of this particular light chain should be obtained under different experimental conditions in particular by using lower ratio of trypsin to S-1. The specific and reproductible action of trypsin on different types of S-1 and the ability of the original chymotryptic S-1 to be fractionnated into two isoenzymes each having a single light chain (4) prompted us to investigate the eventual effects of peptide bond cleavage on the ATPase activities of all the chymotryptic S-1 species. We found that upon trypsin treatment of all of them, the only ATPase activity modified is the actin-stimulate ATPase which is almost completely destroyed. Loss of this particular enzymatic activity during the course of the tryptic digestion of HMM has been previously reported by Perry (22); on the other hand most of the tryptic HMM-S1 preparations reported so far are known to be little or not activated by F-actin althought they still interact reversibly with it (22,23,24). When SDS-

gels are presented for such S-1 preparations, they show in most cases, high amount of heavy chain degradation components of Mr = 74K and 50K (21,25,26). The data we obtained in this work, demonstrate for the first time that the structural integrity of the COOH-terminal region of the heavy chain segment of S-1 at the junction to 75-20K is necessary for preservation of the actin activated ATPase; this is particularly illustrated by the ability of F-actin to afford complete and specific protection of this region against proteolysis and to avoid concomitantly the ATPase inhibition.

During their initial work on the tryptic digestion of heavy meromyosin, Szilagyi et al.(27) noted that cleavage of heavy chain is retarded when actin is present in the reaction mixture. In contrast Balint et al.(25) found later no effect of F-actin on the further fragmentation of HMM by trypsin in the presence of divalent cations; however the banding pattern of the starting HMM material they used (cf.Fig.6 of 25) clearly shows extensive nicking of the heavy chain with appreciable amount of the 74K and 50K fragments. Furthermore actomyosin and acto-HMM complexes remain relatively susceptible to proteolysis because for steric reasons actin does not interact with all heads in these complexes. This is in contrast to the stoechiometric binding of isolated S-1 to actin.

Limited proteolysis of myosin and HMM has been used to probe the submolecular structure of these contractile proteins and other functionnal properties such as binding of Ca^{2+} and Mg^{2+}(28); in this work we show that proteolysis of S-1 is an excellent probe of its interaction with actin and provides for the first time an approach to the characterization of the actin binding site in myosin head. The protection afforded by actin can be a direct or indirect effect. In the first case, at least part of the actin recognition site is at or near the 75K-20K junction. In the second case actin interacts with some site of the heavy chain but induces changes in the COOH-terminalregion. The importance of the implication of this latter domain in actin binding and in actin-activated ATPase must be related to the fact that it is near the hinge portion of myosin, contains the reactive SH_1 and SH_2 (1) and, as recently postulated, at least part of myosin active site (29,30). Finally the "3-pieces" trypsin modified S-1 appears as an excellent material for the study of the structural basis of the activation of Mg ATP hydrolysis by actin.

ACKNOWLEDGMENT : This work is supported by a grant from Direction Générale à la Recherche Scientifique et Technique (n°76.7.1185).

REFERENCES

1. Balint,M., Wolf,I., Tarcsafalvi,A., Gergely,J. and Sréter,F. (1978) Arch.Biochem.Biophys.190, 793-799.

2. Offer,G., Moos,C. and Starr,R. (1973) J.Mol.Biol., 74, 653-676.
3. Weeds,A.G. and Taylor,R.S. (1975) Nature,257, 54-56.
4. Wagner,P.D. and Weeds,A.G.(1977) J.Mol.Biol., 109, 455-473.
5. Straub,F.B. (1942) Stud.Inst.Med.Chem.Univ.Szeged., 3-16.
6. Spudich,J.A. and Watt,S. (1971) J.Biol.Chem., 246, 4866-4871.
7. Godgrey,J.E. and Harrington,W.F. (1970) Biochemistry, 9, 886-893.
8. West,J.J., Nagy,B. and Gergely,J. (1967) J.Biol.Chem., 242, 1140-1145.
9. Mornet,D., Der Terrossian,E., Pradel,L.A., Kassab,R., and Barman,T.E. (1977) Febs Lett., 84, 362-366.
10. Terasaki,W.L. and Brooker,G. (1976) Anal.Biochem., 75, 447-453.
11. Lowry,O.H., Rosebrough,N.J., Lewisfarr,A. and Randall,R.J. (1951) J.Biol.Chem., 193, 265-275.
12. Laemmli,V.K. (1970) Nature, 227, 680-685.
13. Studier,F.W. (1973) J.Mol.Biol., 79, 237-248.
14. O'Farrell,P.H. (1975) J.Biol.Chem., 250, 4007-4021.
15. Porzio,M.A. and Pearson,A.M. (1977) Biochim.Biophys.Acta,490,27-34.
16. White,H.D. and Taylor,E.W. (1976) Biochemistry, 15, 5818-5826.
17. Jacobson,G.R. and Rosenbuch,J.P. (1976) Proc.Nat.Acad.Sci.USA, 73,2742-2746
18. Lu,R., Sosinski,J., Balint,M. and Sréter,F. (1978) Fed.Proc. 37 p.1695.
19. Cardinaud,R.(1977) Proceedings of 6th Meeting of European Muscle club, p.23, Saclay, France.
20. Weeds,A.G. and Frank,G. (1972) Cold Spring Harbor Symp.Quant.Biol.,37, 9-14.
21. Hayashi,Y. (1972) J.Biochem., 72, 83-100.
22. Jones,J.M. and Perry,S.V. (1966) Biochem.J., 100,120-130.
23. Mueller,H. and Perry,S.V. (1962) Biochem.J., 85, 431-439.
24. Yagi,K. (1976) Adv.in Biophys., 8, 1-34.
25. Balint,M., Sréter,F.A., Wolf,I., Nagy,B. and Gergely,J. (1975) J.Biol. Chem., 250, 6168-6177.
26. Cardinaud,R., Dassin,E. and Pelletier,F. (1973) Biochem.Biophys.Res.Comm. 52, 1057-1063.
27. Szilagyi,L., Kurennoy,I., Balint,M. and Biro,E.N.A. (1974) Proceedings of 9th Febs Meeting,31, p.47, Budapest.
28. Weeds,A.G. and Pope,B. (1977) J.Mol.Biol., 111, 129-157.
29. Ramirez,F., Shukla,K.K. and Levy,H.M. (1979) J.Theor.Biol.,76, 351-357.
30. Shukla,K.K., Ramirez,F., Marecek,J.F. and Levy,H.M. (1979) J.Theor. Biol., 76, 359-367.