ISOLATION AND CHARACTERIZATION OF THE TRYPSIN-MODIFIED MYOSIN -S1 DERIVATIVES

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1. Introduction

Tryptic fragmentation of native chymotryptic S1 from skeletal myosin, under controlled conditions in the absence and presence of F-actin, gives rise to 3 possible S1 derivatives the heavy chain of which is a complex of 2 or 3 fragments. These were referred to as (27 k–50 k–20 k)–S1, (27 k–70 k)–S1 and (75 k–22 k)–S1. The mechanism of their formation and their major enzymatic properties have been detailed [1,2]. We have shown, in particular, that actin binding to S1 modulates specifically peptide bond cleavage at a flexible C-terminal 50 k–20 k hinge of the heavy chain and protects against the resulting loss of acto–S1 ATPase activity. In our laboratory, the 3 proteolytic derivatives are serving to investigate the topography of S1 heavy chain–actin interaction with the use of protein crosslinking experiments [2].

Here we describe the isolation in pure state of these S1 species together with some of their interesting structural features including the distribution of the methylated residues over the various tryptic fragments of the heavy chain.

2. Experimental

Rabbit myosin was prepared according to [3]. Subfragment-1 (S1) was prepared by digestion of myosin filaments with chymotrypsin [4], purified on Sephacryl S-200 in 0.05 M Tris–HCl buffer (pH 7.5) and resolved into pure S1A1 and S1A2 isoenzymes by ion-exchange chromatography [4]. Actin was purified according to [5]. Protein concentrations for S1 and actin were estimated using $E^{1\%,\,1\ cm}_{280\ nm}$ = 7.5 [6] and 11.0 [7], respectively. The concentration of proteolytic S1 derivatives was determined according to [8] using bovine serum albumine as standard. The same procedure was used for the purified peptides. SDS–12.5% polyacrylamide slab gel electrophoresis was done as in [1]. ATPase activities were determined as in [9]. α-Chymotrypsin and TPCK-trypsin were from Worthington. ε-*N*-monomethyllysine and ε-*N*-trimethyllysine were from Calbiochem; 3-methylhistidine was from Sigma.

2.1. *Isolation of fragmented S1 derivatives*

The preparative isolation of trypsin-modified S1 derivatives was conducted as follows:

The (27 k–50 k–20 k)–S1 derivative: S1 at 2 mg/ml in 0.1 M $KHCO_3$ was treated at 25°C for 30 min with trypsin added at a protease/S1 ratio (w/w) of 1:100. The reaction was terminated by adding soybean trypsin inhibitor in an amount twice that of trypsin (w/w); the digest (20 ml) was immediatly filtered on a Sephacryl S-200 column (3 × 70 cm) eluted at 4°C with 50 mM NH_4HCO_3 (pH 8); this yielded a S1 with a heavy chain cut into 3 fragments of M_r = 27 000 (27 k), 50 000 (50 k) and 20 000 (20 k).

The (27 k–70 k)–S1 derivative: The acto–S1 complex (molar ratio F–actin:S1 = 2) was digested using essentially the same experimental procedure as for S1 alone. The reaction was stopped by addition of trypsin inhibitor and the digest was adjusted to 0.5 M KCl, 15 mM $NaPP_i$, 1 mM $MgCl_2$ and 50 mM Tris–HCl (pH 8.0); after centrifugation for 2 h at 110 000 × *g*, 4°C, the clear supernatant was subjected to Sephacryl S-200 gel filtration. This resulted in the obtention of a second S1 derivative with a heavy chain composed of only 2 fragments of M_r = 27 000 (27 k) and 70 000 (70 k).

The (75 k–22 k)–S1 derivative: S1 (2 mg/ml) in 0.1 M $KHCO_3$ (pH 8.0) was digested at 25°C for

15 min with a trypsin/S1 ratio (w/w) of 1:1500. After the stopping of proteolysis and purification of the reaction mixture by Sephacryl S-200 gel filtration a third proteolytic S1 derivative was obtained the heavy chain of which was cleaved into 2 fragments of M_r = 75 000 (75 k) and 22 000 (22 k).

2.2. *Isolation of the tryptic fragments of S1 heavy chain*

The polypeptides 75 k, 70 k, 50 k, 27 k and 20 k which were components of the heavy chain of the various trypsin-modified S1 derivatives as well as the intact 95 k heavy chain of native S1 were readily separated by preparative electrophoresis in 0.1% SDS–12.5% polyacrylamide slab-gels using the procedure reported in [10] with slight modifications. In the case of the 75 k fragment which was essentially an intermediary proteolytic component [1], it was prepared in sufficient quantity by submitting to electrophoresis the digestion mixture described above for the preparation of the (27 k–50 k–20 k)–S1 derivative except that proteolysis was stopped after a critical reaction time of 15 min instead of 30 min. Isolation in high amount of pure 50 k and 27 k peptides was performed by the column procedure in [11].

2.3. *Amino acid analysis*

Amino acid analyses were performed on protein samples hydrolyzed at 110°C under vacuum in 5.7 N HCl for 24 h and 48 h using 2 amino acid analyzers. Conventional analyses were done in a Beckman analyzer (Model 119B). Cysteine was estimated as cysteic acid [12]. The identification and determination of methylated lysine and methylated histidine derivatives were performed independently with a laboratory-built, high-performance amino acid analyzer equipped with a fluorometric detection system operating with the *O*-phtaldialdehyde–β-mercaptoethanol reagent [13]. A single resin bed (0.50 X 27 cm) of the cation exchanger Aminex A6 (Bio Rad) was used and was eluted with 0.4 M sodium citrate at a flow rate of 27 ml/h. The separation of 3-methylhistidine was obtained by eluting at pH 4.20 with a column temperature at 50°C. The resolution of the methylated lysine derivatives was achieved by column elution at pH 6.9 and at 29°C. Acid hydrolysates were analyzed with each of these 2 elution systems.

3. Results and discussion

3.1. *Characterization of the trypsin-modified S1 derivatives*

The proteolytic derivatives isolated by gel filtration after limited tryptic digestion of S1 and of its complex with actin were characterized as to their homogeneity, stability and, in particular, the compositional properties of their heavy chain tryptic fragments were established. As indicated in fig.1, the (27 k–50 k–20 k)- and (27 k–70 k)-trypsin modified S1 derivatives, 2 major protein materials, are quite homogenous as analyzed by gel electrophoresis (lanes e,i). When the starting enzyme is pure S1A2 no minor bands migrating at both sides of the 20 k position are present in the gel. Neither the gel pattern nor the remaining ATPase activities of the (27 k–50 k–20 k)–S1 derivative [1] were changed upon further treatment with a trypsin/protein ratio (w/w) of 1:100 for 120 min at 25°C (pH 8.0). This indicates that the 3 heavy chain fragments are relatively resistant to trypsin. As the actin and nucleotide binding sites in myosin heads are functionally interdependent [14–16], it was obviously of interest to test the ability of actin to protect the intact ATPase site of this S1 derivative against its eventual inactivation by a high amount of trypsin and to probe the influence of peptide bond cleavage at the 75 k–20 k junction on the interactions linking the actin and nucleotide sites; for

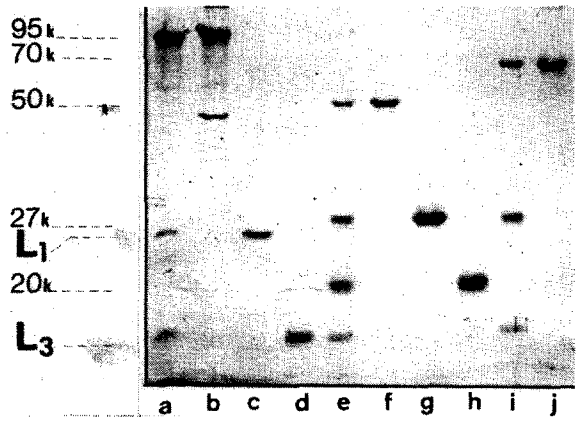

Fig.1. Purified trypsin-modified S1 derivatives and heavy chain tryptic-fragments. 0.1% SDS–12.5% polyacrylamide slab gel electrophoretic analysis: (a) native S1 A1 + A2; (b) S1 heavy chain; (c) light chain 1; (d) light chain 3; (e) (27 k–50 k–20 k)–S1A2; (f) 50 k peptide, (g) 27 k peptide; (h) 20 k peptide; (i) (27 k–70 k)–S1A2; (j) 70 k peptide.

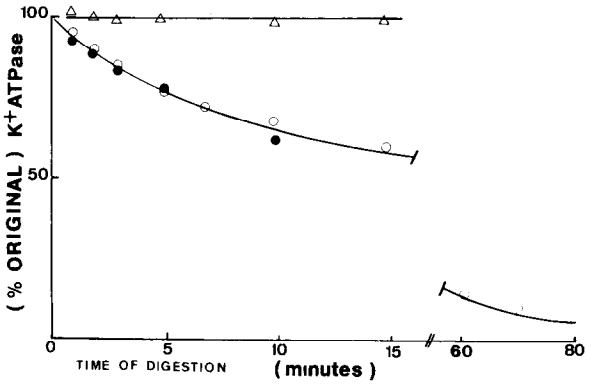

Fig.2. Effect of actin on the time course of changes in the K^+-ATPase activities of native S1 and (27 k–50 k–20 k)–S1 derivative during digestion with trypsin. S1 A1 + A2 (20 μM) was incubated in 100 mM CO_3HK (pH 8.0), 25°C, with a trypsin/enzyme ratio (w/w) of 1:5 in the absence (○) and presence (△) of actin (40 μM). (27 k–50 k–20 k)–S1 derivative (20 μM), freshly prepared as indicated in section 2, was treated with trypsin as above in the presence of 40 μM actin (●). At the times indicated samples were taken from the digestion mixtures and assayed for K^+-ATPase activities. The value of the original K^+-ATPase of native and trypsin-modified S1 was 3.5 μmol . mg^{-1} . min^{-1}.

comparison native S1 was submitted to the same treatment. As shown in fig.2 a progressive abolition of the K^+-ATPase (and also of the Ca^{2+}-ATPase) was obtained by treating native S1 with a trypsin/enzyme ratio (w/w) of 1:5 at 25°C (pH 8.0) for 60–80 min. This result is in agreement with the report [17] describing the rapid inactivation of the K^+-ATPase of myosin upon reaction of the protein with a similar amount of trypsin. As evidenced in fig.2, the addition of actin to the digestion medium resulted in the total protection of the K^+- and Ca^{2+}-ATPases. Thus, despite the high amount of trypsin employed, actin was efficient in altering also the proteolytic susceptibility of the ATPase site of native S1. In contrast, fig.2 reveals that actin was not able to protect the (27 k–50 k–20 k)–S1 the K^+-ATPase of which was lost at the same rate as that of native S1 whether actin was present or not in the reaction mixture. There is no doubt that actin does reversibly bind to the S1 derivative under the experimental conditions as assessed by separate turbidimetric measurements [1] and by the chemical crosslinking of actin to 50 k and 20 k fragments of the S1 derivative [2]. The data suggest that the structural integrity of the 75 k–20 k junction may be essential for allowing actin attachment to S1 to be specifically transmitted to the nucleotide site.

The (27 k–70 k)–S1 derivative is relatively unstable and during handling of this material even in the cold partial cleavage of the 70 k peptide into 50 k and 22 k components is often observed. The 22 k element is a precursor of the stable 20 k peptide and contains a protease-sensitive NH_2-terminal 2 k segment connecting 50 k and 20 k domains [2]; the tryptic degradation of this segment is intimatly associated with the alteration of the acto–S1 ATPase [2]. The accumulation of the transient (75 k–22 k)–S1 derivative (fig.3) upon treatment of S1 with a protease/enzyme ratio as low as 1:1500 provides a unique way for isolation of the 22 k peptide and therefore for structural characterization of the critical 50 k–20 k junction.

3.2. *Characterization of the heavy chain tryptic fragments*

The purity of most of the heavy chain tryptic peptides we have isolated is also illustrated in fig.1; their compositional properties are presented in table 1 in comparison with the amino acid composition of the intact 95 k heavy chain. To search for eventual differences that may exist between the 2 S1 isoenzymes at the level of the elementary chemical structure of their heavy chain, analyses were performed on peptides

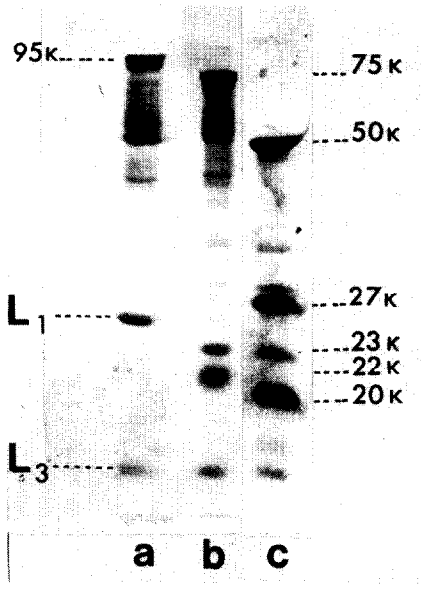

Fig.3. Electrophoretic analysis of the transient (75 k–22 k)-trypsin-modified S1 derivative: (a) native S1 (A1 + A2); (b) purified (75 k–22 k)–S1 (A1 + A2) derivative; (c) purified (27 k–50 k–20 k)–S1 (A1 +A2) derivative. The 23 k protein band is a degradation product of light chain 1 [1].

Table 1
Amino acid composition of the peptides derived from limited tryptic fragmentation of chymotryptic S1 heavy chain[a]

Amino acid	95 k	75 k	70 k	50 k	27 k	20 k
Lys	67.5	48.0	52.0	32.0	15.6	13.7
His	18.5[b]	14.0	13.5[b]	9.7	5.2	5.5[b]
Arg	30.0	21.0	21.5	10.5	8.0	9.0
Asp	75.5	56.5	55.0	39.0	21.8	16.2
Thr[c]	51.0	38.5	33.5	25.7	12.8	8.7
Ser[c]	40.5	35.0	30.0	21.6	17.5	10.3
Glu	100.5	86.5	81.0	55.0	24.0	22.7
Pro	29.0	22.0	18.0	10.3	10.2	5.5
Gly	62.5	47.0	43.5	32.5	18.0	13.6
Ala	62.5	51.5	46.5	32.3	17.4	11.7
1/2 Cys[d]				4.0	1.8	2.2
Val[e]	50.5	38.5	38.0	27.2	14.2	10.2
Met	24.0	16.8	15.5	11.0[f]	7.0[f]	4.2[f]
Ile[e]	43.5	31.5	27.0	24.0	12.0	7.7
Leu	63.0	43.0	53.0	36.6	12.7	15.5
Tyr	29.0	23.5	18.5	18.2	9.0	3.5
Phe	41.5	36.0	30.5	24.0	8.7	7.0

[a] All the experimental values given are av. ⩾6 determinations made on peptides issued from S1A1 as well as S1A2 following 24 h and 48 h hydrolyses except when noted. Values are expressed as residues/peptide using the indicated app. M_r-values.
[b] Not corrected for the presence of 1 residue of 3-methylhistidine not resolved from histidine with the standard Beckman program (see table 2)
[c] Not corrected for hydrolytic losses
[d] Analysed as cysteic acid
[e] From the 48 h hydrolysis value
[f] Analyzed as methionine sulfone

issued from both S1A1 and S1A2. No peptide was found bearing significant differences related to its origin. Except the lower amount found for half-cystine residues (2–3 instead of 4) the amino acid composition of the 20 k material is, within experimental error, in good agreement with the entire covalent structure of the corresponding peptide issued from tryptic hydrolysis of HMM [18].

A remarkable structural feature of myosin heads from rabbit fast skeletal muscle is the presence in the heavy chain of ε-*N*-monomethyllysine, ε-*N*-trimethyllysine and 3-methylhistidine in a ratio of ~1:2:1 [19,20]. Specific amino acid analyses of these unusual residues allowed us to determine their exact distribution over the various tryptic fragments and to characterize further the S1 heavy chain in regard to this class of side chains the possible biological role of which is still unknown. As illustrated in table 2, each peptide presented a so well-defined pattern of methylation that it is a simple matter to identify unambiguously any fragment after determination of the methylated amino acid(s) associated with it. Thus, the NH_2-terminal 27 k peptide contained the single monomethyllysine as well as one of the 2 trimethyllysine residues initially present in the 95 k heavy chain; the second trimethyllysine derivative of the protein is within the 50 k fragment. The C-terminal 20 k peptide contained only the unique 3-methylhistidine [18]. The composition of the overlapping 70 k and 75 k peptides provided a further confirmation of this distribution. In agree-

Table 2
Content of methylated lysine and histidine residues in the peptides derived from limited tryptic cleavage of chymotryptic S1 heavy chain[a]

Amino acid	95 k	70 k	50 k	27 k	20 k	75 k
3-Methylhistidine	1.0–1.2	1.0–1.2	0	0	1.0–1.2	0
ε-*N*-Monomethyllysine[b]	0.5–0.7	0	0	0.5–0.7	0	0.5–0.7
ε-*N*-Trimethyllysine[c]	2.0–2.3	1.0–1.2	1.0–1.2	1.0–1.2	0	2.0–2.2

[a] Experimental values are based on at least five 24 h hydrolysates and are expressed as residues/peptide assuming the indicated app. M_r values. Both the minimal and maximal values found are given. Each peptide analyzed was originated from both S1A1 and S1A2
[b] Calculated relative to phenylalanine and histidine residues
[c] Calculated relative to histidine residues

ment with the original findings of [19] less than stoichiometric amount of monomethyllysine was observed in every fragment containing this residue in contrast with the stoichiometric values found under identical experimental conditions for the other derivatives. This substoichiometry is not related to the different light chain subunit structure of S1A1 and S1A2 as similar results were obtained for material issued from both isoenzymes; it is not due to hydrolytic destruction as this amino acid was quantitatively recovered after 48 h acid hydrolysis in the absence and presence of S1; finally it cannot be due to loss of some peptide component, containing the monomethylated lysine, from the proteolytically prepared S1, as suggested in [19], since this residue is located not in the protease-sensitive C-terminal region of the heavy chain, as supposed [19], but rather in the NH_2-terminal portion of the polypeptide. The results can be accounted for only by a fractional methylation of the 27 k segment as frequently observed for other methylated proteins [21]. The two methyllysine-containing fragments 27 k and 50 k bear functional features; the 27 k element contains ligands at or near myosin ATPase site [11, 22] while the 50 k unit participates in action attachment to S1 [2,23].

The presence of a single trimethyllysine residue in each of the 27 k and 50 k fragments offers the interesting possibility to probe specifically for the first time the effects of actin or nucleotide binding to S1 on these particular regions of the heavy chain by exploiting the ^{13}C NMR properties of the ϵ-*N*-trimethylammonium group [24].

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References

[1] Mornet, D., Pantel, P., Audemard, E. and Kassab, R. (1979) Biochem. Biophys. Res. Commun. 89, 925–932.
[2] Mornet, D., Bertrand, R., Pantel, P., Audemard, E. and Kassab, R. (1981) Biochemistry, in press.
[3] Offer, G., Moss, C. and Starr, R. (1973) J. Mol. Biol. 74, 653–676.
[4] Weeds, A. G. and Taylor, R. S. (1975) Nature 257, 54–56.
[5] Spudich, J. A. and Watt, S. (1971) J. Biol. Chem. 246, 4871–4886.
[6] Wagner, P. D. and Weeds, A. G. (1977) J. Mol. Biol. 109, 455–473.
[7] West, J. J., Nagy, B. and Gergely, J. (1967) J. Biol. Chem. 242, 1140–1145.
[8] Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275.
[9] Mornet, D., Pantel, P., Audemard, E. and Kassab, R. (1979) Eur. J. Biochem. 100, 421–431.
[10] Van De Kerckhove, J. and Van Montagu, M. (1974) Eur. J. Biochem. 44, 279–288.
[11] Mornet, D., Pantel, P., Bertrand, R., Audemard, E. and Kassab, R. (1980) FEBS Lett. 117, 183–188.
[12] Hirs, C. H. W. (1967) Methods Enzymol. 11, 59–62.
[13] Lee, K. S. and Drescher, D. G. (1978) Int. J. Biochem. 9, 457–467.
[14] Beinfeld, M. C. and Martonosi, A. N. (1975) J. Biol. Chem. 250, 7871–7878.
[15] Highsmith, S. (1976) J. Biol. Chem. 251, 6170–6172.
[16] Marston, S. B., Tregar, R. T., Rodger, C. D. and Clarke, M. L. (1979) J. Mol. Biol. 128, 111–126.
[17] Yamashita, T. and Mimura, T. H. (1974) J. Biochem. (Tokyo) 76, 1049–1059.
[18] Gallagher, M. and Elzinga, M. (1980) Fed. Proc. FASEB 39, 2168.
[19] Kuehl, W. M. and Aldelstein, R. S. (1969) Biochem. Biophys. Res. Commun. 37, 59–65.
[20] Huszar, G. and Elzinga, M. (1969) Nature 223, 834–835.
[21] Paik, W. K. and Kim, S. (1979) Adv. Enzymol. 42, 227–286.
[22] Szilagyi, L., Balint, M., Streter, F. A. and Gergely, J. (1979) Biochem. Biophys. Res. Commun. 87, 936–945.
[23] Yamamoto, K. and Sekine, T. (1979) J. Biochem. (Tokyo) 86, 1863–1868.
[24] Wilbur, D. J. and Allerhand, A., (1977) FEBS Lett. 74, 272–274.