Proc. Natl. Acad. Sci. USA
Vol. 77, No. 4, pp. 2010–2013, April 1980
Biochemistry

Identification of a region susceptible to proteolysis in myosin subfragment-2

(enzymatic cleavage/hinge region)

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Communicated by Manuel F. Morales, January 28, 1980

ABSTRACT **Comparison of the NH_2-terminal sequence of myosin short subfragment-2 (M_r of subunit = 37,000) and long subfragment-2 (M_r of subunit = 59,000) demonstrates that the former represents the NH_2-terminal portion of the latter and suggests that the hinge region in myosin rod is in the COOH-terminal portion of the long subfragment-2.**

Myosin molecules are two-headed structures, each head corresponding to an approximately 90-kilodalton (kDal) segment of the NH_2-terminal portion of a 200-kDal chain folded in a globular shape; over the rest of their length the two chains are joined in a coiled-coil α-helical rod (1). The globular head and part of the α-helical rod form the crossbridges that connect the core of the myosin filaments with the actin filaments (2). Two hinge-like regions have been postulated in the myosin molecule, one at the junction of the heads and the α-helical rod, and one within the rod, where the connecting link joins the core of the thick filaments. These hinges play a crucial role in molecular models of muscle contraction, permitting the movement of the heads on actin and of the whole crossbridge with respect to the myosin core (3, 4). Two proteolytic fragments of myosin, subfragment-1 (S-1) and subfragment-2 (S-2), have been identified with the head and connecting link portion, respectively (1). Whereas the location of the S-1/link hinge can be fixed with a fair degree of certainty at the junction of S-1 and S-2, the location of a second hinge in relation to the rod is not fully established. It has been suggested that regions in the myosin molecule that exhibit higher susceptibility to proteolytic enzymes correspond to the hinge region (3–9). Because S-2 has recently been found in two forms—a longer one with a subunit chain weight of 59 kDal (10–12), containing a flexible region (11), and a shorter one with a chain weight about 37 kDal—it appeared to be of interest to clarify the relationship of the two S-2 fragments. It would be reasonable to assume that the region of the long S-2 with higher proteolytic susceptibility would be the hinge region. Our results indicate that the short S-2 arises from the NH_2-terminal region of the long S-2, adjacent to S-1, and the presumptive hinge region of high proteolytic susceptibility is in the COOH-terminal part of the long S-2.

MATERIALS AND METHODS

Myosin was prepared from rabbit back muscle (13). Trypsin (TRTPCK) and chymotrypsin (CDI) were purchased from Worthington and twice-crystallized papain from Sigma; Sephacryl S-200 was obtained from Pharmacia; all chemicals used were reagent or Sequenal grade. Polyacrylamide gel electrophoresis in $NaDodSO_4$ was carried out according to Weber and Osborn (14).

Short S-2. Heavy meromyosin (HMM) was prepared by digesting myosin with trypsin at an enzyme-to-substrate ratio of 1:500 (wt/wt) for 5 min. After removal of light meromyosin (LMM) and undigested myosin, HMM was again subjected to tryptic digestion with an enzyme-to-substrate ratio of 1:50 for 60 min at room temperature in a solution containing 30 mM Tris·HCl at pH 8.0, 10 mM 2-mercaptoethanol, and 2 mM EDTA (13). Soybean trypsin inhibitor (twice the weight of trypsin) was added to stop the reaction, and the digests were lyophilized immediately. The digests were redissolved in 50 mM Tris·HCl at pH 8.0 and 6 M guanidinium chloride (GdmCl), alkylated with iodoacetic acid (15), and then extensively dialyzed against distilled water.

Long S-2. Three kinds of long S-2 were prepared. S-2 (HMM-C) was prepared from chymotryptic digests of HMM. S-2 (Rod-C) and S-2 (Rod-P) were prepared from the rod that had been obtained by chymotrypsin or papain digestion of myosin, respectively. The details of the procedures are given below.

S-2 (HMM-C). Myosin was digested with chymotrypsin at an enzyme-to-myosin ratio of 1:200 (wt/wt) for 15 min in a solution containing 0.5 M KCl, 50 mM Tris·HCl at pH 8.0, 1 mM dithiothreitol, and 2 mM $CaCl_2$. After removal of LMM and undigested myosin, HMM in the supernatant solution was further digested with chymotrypsin at an enzyme-to-HMM ratio of 1:100 (wt/wt) for 20 min in 50 mM Tris·HCl, pH 8.0/0.14 M KCl/1 mM EDTA (10). S-2 was separated from S-1 after ethanol precipitation of the digests (16).

S-2 (Rod-C). Myosin was digested with chymotrypsin at an enzyme-to-substrate ratio of 1:200 (wt/wt) for 20 min in 10 mM sodium phosphate, pH 7.0/0.12 M KCl/1 mM dithiothreitol/1 mM EDTA. Under these conditions the cleavage occurs at the S-1/rod junction (10). After purification by ethanol precipitation the rod was subjected to trypsin digestion in 50 mM sodium phosphate, pH 6.2/0.5 M KCl (11) with a trypsin-to-rod ratio of 1:400 (wt/wt) for 5 min. S-2 was separated from LMM on the basis of its solubility in low ionic strength buffer (10 mM sodium phosphate/30 mM KCl).

S-2 (Rod-P). The rod portion was prepared after papain digestion according to Bálint *et al.* (17). S-2 (Rod-P) was produced by subsequent chymotryptic digestion in 0.5 M KCl/10 mM sodium phosphate, pH 7.0, at an enzyme-to-substrate ratio of 1:200 (10).

Edman degradations were carried out by using a Beckman 890C sequencer operated with program 122974. Anilinothiazolinones were converted to corresponding phenylthiohydantoins and identified by two independent methods: thin-layer chromatography (18) and amino acid analysis after regeneration of free amino acids by hydrolysis in 56.6% (wt/wt) hydriodic acid at 150°C for 4 hr (19).

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Abbreviations: kDal, kilodaltons; HMM, heavy meromyosin; LMM, light meromyosin; S-1, subfragment-1; S-2, subfragment-2; GdmCl, guanidinium chloride.

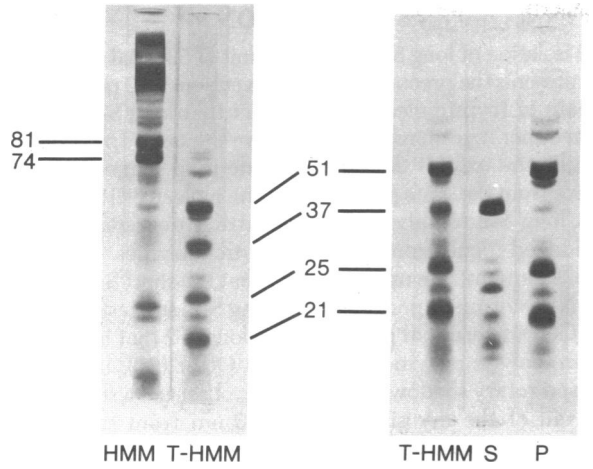

FIG. 1. Polyacrylamide gel electrophoresis of HMM and tryptic digests of HMM (T-HMM); the two gels on the left are 6% and the three gels on the right are 7.5% gels. Gels S and P, supernatant and pellet, respectively, after digestion and alkylation. Masses in kDal are indicated.

RESULTS

Purification of S-2 Preparations. After alkylation and removal of GdmCl, short S-2, designated as S-2(S), as well as some minor components with smaller molecular weights, remained in the supernatant (Fig. 1, gel S), whereas the three fragments arising from the head portion became insoluble (Fig. 1, gel P). The material eluted as the first peak upon gel filtration on a Sephacryl S-200 column (Fig. 2) has an extremely low extinction coefficient at 280 nm; it represents mainly the 37-kDal fragment, as shown by $NaDodSO_4$ gel electrophoresis (*Inset*, Fig. 2). Fractions containing the 37-kDal fragments were collected, pooled, lyophilized, and used for further studies. The homogeneity of the preparation is illustrated in Fig. 3.

Because the mobility on $NaDodSO_4$ gels of the long S-2 prepared from the myosin rod appears to be the same as that of S-2 (HMM-C) obtained from HMM, we used the latter for most of the studies on long S-2. Routinely, crude S-2 (HMM-C)

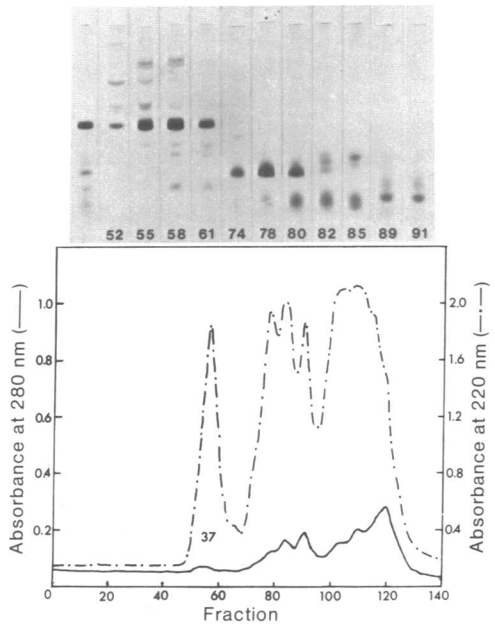

FIG. 2. Gel filtration of a short S-2 preparation on a 200 × 1.9 cm column of Sephacryl S-200 in 0.1 M ammonium bicarbonate at 23°C. The fraction size was 4 ml. The numbers on the gels indicate the fractions from which samples were taken for gel electrophoresis. The 37-kDal peak is indicated.

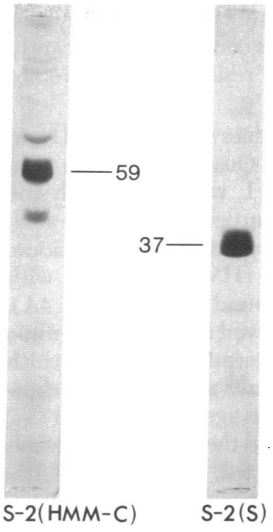

FIG. 3. A 7.5% polyacrylamide gel electrophoresis of purified long S-2 (HMM-C) and short S-2 (S). Masses in kDal are indicated.

was alkylated immediately after preparation in order to avoid further enzymatic degradation and was then passed through a Sephacryl S-200 column (results not shown, conditions same as Fig. 2) to remove contaminating peptides with smaller molecular weight. Purified S-2 (HMM-C) contained no more than 5% contaminating peptides (Fig. 3); this level of contamination poses no problem in sequence determination.

Amino Acid Compositions of Long and Short S-2. The amino acid compositions of S-2 (S) and S-2 (HMM-C) (Table 1) are quite similar. Both are rich in residues that are known to favor helix formation, such as lysine, glutamic acid, alanine, and leucine; on the other hand, both lack, or contain only very small amounts of, histidine, proline, and glycine, residues that are not favorable for helix formation (20). The results are consistent with the view that both long and short S-2 are part of the α-helical portion of myosin and are in good agreement with the

Table 1. Amino acid compositions of long S-2, short S-2, and difference peptide*

Amino acid residue	Long S-2 59 kDal Residues	Long S-2 59 kDal Mole %	Short S-2 37 kDal Residues	Short S-2 37 kDal Mole %	Difference peptide Residues	Difference peptide Mole %
Lys	82	15.9	48	15.0	34	13.7
His	4	0.8	2	0.7	2	1.0
Arg	23	4.4	12	3.6	11	2.1
Cys†	3	0.5	2	0.5	1	0.5
Asp	46	8.9	31	9.6	15	7.6
Thr	21	4.1	14	4.4	7	3.5
Ser	16	3.1	8	2.5	8	4.0
Glu	147	28.6	95	29.8	52	26.5
Pro	—	—	—	—	—	—
Gly	9	1.7	4	1.4	5	2.6
Ala	60	11.6	32	10.1	28	14.3
Val	15	3.0	10	3.0	5	2.6
Met	10	2.0	6	2.0	4	2.0
Ile	17	3.3	12	3.8	5	2.6
Leu	59	11.5	41	12.9	18	9.2
Tyr	—	—	—	—	—	—
Phe	3	0.6	2	0.7	1	0.5
Total	515		319		196	

* Samples were hydrolyzed for 20–22 hr in 6 M HCl at 110°C. Values for long S-2 are obtained from S-2 (HMM-C), and the composition of the difference peptide is obtained by subtracting value of short S-2 from that of long S-2.

† Measured as carboxymethylcysteine.

previously published data on short S-2 (1). It is noteworthy that the segment susceptible to proteolysis contains a significantly higher fraction of glycine and serine residues, which are not favorable for helix formation.

Amino Acid Sequence. Sixteen residues were identified from the NH_2-terminus of S-2 (S) (Fig. 4A). Quantitative yields were obtained at each step except at step 1, which yielded lysine (about 60%) and small amounts of aspartic acid, glutamic acid, and alanine. This suggests microheterogeneity at this position. The NH_2-terminal sequence of S-2 (HMM-C) begins with Leu-Lys, followed by the sequence found in S-2 (S) (Fig. 4A). A lower yield of lysine was also observed at step 3; the amino acid liberated corresponds to the NH_2-terminus of S-2 (S), which again reveals heterogeneity. The results unequivocally show that the tryptic and chymotryptic cleavage sites in the S-1/S-2 junction are only two residues apart and the short S-2 represents the NH_2-terminal moiety of the long S-2.

Determining the amino acid sequence of the long S-2 prepared from chymotryptic rod [S-2 (Rod-C)] gave results identical to those obtained with S-2 (HMM-C). However, the first step of S-2 (Rod-P) yielded lysine and the subsequent ones liberated successive pairs of amino acids corresponding to neighbors in the sequence of S-2, such as Lys + Ala, Ala + Glu, Glu + Thr, etc. The results are consistant with the sequence shown in Fig. 4A, assuming that S-2 (Rod P) can result from cleavage by papain of either the Leu-Lys or the Lys-Lys bond (Fig. 4B).

DISCUSSION

The isolation of long S-2 with a subunit of 59 kDal suggests that it represents the precursor of the conventional S-2 resulting from papain or trypsin cleavage. The fact that long S-2s prepared from either myosin rod or HMM have the same apparent chain weight and exhibit the same peptide map suggests that both derive from the same proportion of the molecule (10). It appears that long S-2 readily undergoes further degradation during prolonged incubation with proteolytic enzymes. The present results demonstrate unequivocally that the short S-2 represents the NH_2-terminal portion of the long S-2, suggesting that it is the COOH-terminal portion of the long S-2 that is susceptible to proteolytic enzymes. Elliott and Offer (21), using an improved rotary shadowing technique, observed a sharp bend in the tail of the myosin molecule 43 nm from the head/tail junction. Takahashi (22), using a negative staining method, reported a bend in the myosin tail located about 65 nm from the end of the tail; bends are also observable in his pictures, as pointed out by Elliott and Offer, between 40 and 50 nm from the head/tail junction. If both long and short S-2 are coiled-coil α-helical structures, their lengths would be 72.0 and 45.6 nm, respectively, assuming the length per residue is the same as in the coiled-coil tropomyosin structure, namely, 0.14 nm (23, 24). Thus it appears that the first bend corresponds to the COOH-terminus of the short S-2 and the second bend could fall in the region corresponding to the peptide that would make up the difference between the long and short S-2.

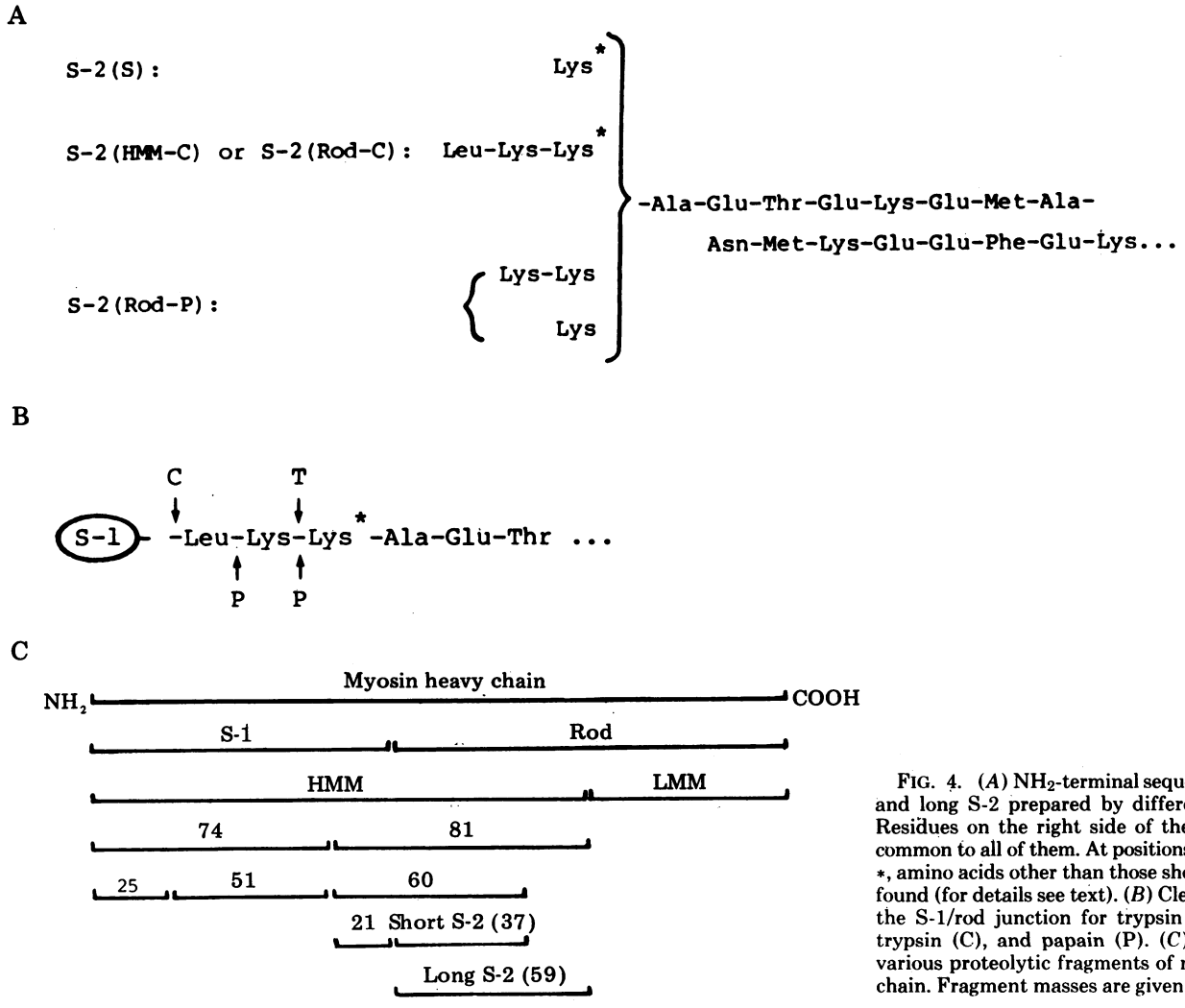

FIG. 4. (A) NH_2-terminal sequences of short and long S-2 prepared by different enzymes. Residues on the right side of the bracket are common to all of them. At positions indicated by *, amino acids other than those shown were also found (for details see text). (B) Cleavage sites in the S-1/rod junction for trypsin (T), chymotrypsin (C), and papain (P). (C) Diagram of various proteolytic fragments of myosin heavy chain. Fragment masses are given in kDal.

Highsmith *et al.* (11) have shown the flexibility of S-2 by measuring the relaxation times of the rod, S-2, and LMM. However, they could not distinguish between S-2 being flexible along its entire length or its possessing a single restricted flexible region. If the flexibility is indeed associated with susceptibility to proteolytic enzymes, our studies suggest that it is the COOH-terminal segment of S-2 that is flexible.

Bálint *et al.* (13) reported that heavy meromyosin obtained by tryptic digestion of myosin contains two main polypeptides whose masses were estimated as 81 and 74 kDal from Na-DodSO$_4$/polyacrylamide gel electrophoresis. Upon further digestion with trypsin, four major fragments with masses of 51, 37, 25, and 21 kDal are formed (Figs. 1 and 4*C*). On the basis of the time course of the appearance of the fragments the following relationship emerged: 81 kDal → 60 kDal → 37 kDal + 21 kDal; 74 kDal → 51 kDal + 25 kDal (13). The 37-kDal fragment, which is considered to be a α-helical by the criterion of resolubilization after precipitation with ethanol, apparently is the subunit of short S-2. However, it remained unclear whether the missing piece during the 81 kDal → 60 kDal transformation arises from the head (NH_2-terminus of 81 kDal) or rod (COOH-terminus of 82 kDal) portion. Because the NH_2-terminal sequence of 37 kDal is identical to that of the long S-2, it becomes obvious that the short fragment lost is located at the COOH-terminus of the 81-kDal fragment or HMM. Furthermore, the long S-2 prepared from HMM seems to be identical with the long S-2 obtained from the rod, and the cleavage sites for chymotrypsin, trypsin, or papain are only two residues apart (Fig. 4*B*). This suggests that there is a rather restricted area in the head/tail junction that is available to proteolytic enzymes. This view is further supported by the fact that the heavy chains of S-1 are usually quite homogeneous. Final settlement of this question will require determining the amino acid sequence of the COOH-terminus of S-1 prepared by different enzymes and comparison of the putative abutting portions of S-1 and S-2 with a corresponding sequence in the 60-kDal fragment of HMM (referred to above and in Fig. 4*C*).

Values of $\langle P_\alpha \rangle$ and $\langle P_\beta \rangle$, parameters of α-helical and β-structure character calculated according to Chou and Fasman (25), for the 19-residue NH_2-terminal segment are 1.25 and 0.78, respectively. These values establish the α-helical character of this region, sinces the criteria for α-helicity are $\langle P_\alpha \rangle > \langle P_\beta \rangle$ and $\langle P_\alpha \rangle \geq 1.03$.

Microheterogeneity of myosin has been documented for the NH_2-terminal portions of the molecule (26), and our results show that heterogeneity exists in the rod portion as well. It is unlikely that the multiple amino acids found in the first position of the sequence of the short S-2 and position three of the long S-2 (Fig. 4*A*) are due to the presence of contaminating peptides, because only a single amino acid was formed at all the other positions in the sequence examined. Nevertheless, the nature of the microheterogeneity is not as yet clear; thus it cannot be decided at present whether the rod of one myosin molecule consists of two identical subunits or not.

Sutoh *et al.* (12) have reported that the long S-2 self-associated under physiological ionic strength, but not the short S-2. In the light of our results it appears that the COOH-terminal portion of the long S-2 is necessary for the association. Comparison of amino acid compositions of the long and the short S-2 suggests, as shown above, that the difference peptide may have less of a helical structure and may play a role both in the flexibility and in the aggregation of S-2. The conclusion on this point will have to await the determination of the amino acid sequence of the relevant regions.

I am grateful to Drs. John Gergely and Manuel Morales for their encouragement and valuable discussions. I also thank Jan Sosinski, Anna Wong, and Sandy Tong for excellent technical assistance and Sally Williams for preparing the manuscript. This work was supported by grants from the National Institutes of Health (AM00485, GM24666, HL5949, and 5S07 RR05711), the National Science Foundation (PCM 7712173), the American Heart Association (781027), and the Muscular Dystrophy Association of America.

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