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Proteolytic Fragmentation of Bovine Heart Heavy Meromyosin*

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ABSTRACT: Bovine heart subfragment 1 was isolated from the heavy meromyosin by digestion with papain. This subfragment 1 appeared as a single peak after Sephadex G-200 chromatography and in the analytical ultracentrifuge. Its molecular weight was 110,000 ± 10,000 as determined by the Yphantis method. The Ca^{2+}–adenosine triphosphate phosphohydrolase activity of this subfragment 1 was 1.6-fold higher than the original heavy meromyosin and 2.4-fold higher than the original myosin in the range of pH 6–9. In contrast to the papain digestion of bovine heart heavy meromyosin, no significant quantities of subfragment 1 could be isolated when trypsin or chymotrypsin digestions were performed under various conditions. Trypsin digestion at pH 7.6, 25° for 30 min did not release subfragment 1 and the Ca^{2+}–ATPase activity was lost. Comparative pH-Stat studies showed that bovine heart heavy meromyosin was more resistant to digestion by papain, chymotrypsin, or trypsin than rabbit skeletal heavy meromyosin. Furthermore, bovine heart myosin was also more resistant to trypsin digestion than was rabbit skeletal myosin. Results from sedimentation studies, the Ca^{2+}–ATPase measurements, and quantitation data obtained from chromatography of the papain digests indicated that bovine heart myosin and heavy meromyosin are composed of two subunits of subfragment 1.

In the past few years, the isolation and characterization of subfragment 1, the enzymically active subunit of rabbit skeletal myosin, has been studied extensively (see *e.g.*, Mueller and Perry, 1962; Young *et al.*, 1965; Kominz *et al.*, 1965; Jones and Perry, 1966; Slayter and Lowey, 1967; Lowey *et al.*, 1969). However, little is known about the preparation of subfragment 1 from other myosins. Previous work has revealed that heart myosin differs from skeletal myosin with respect to its ATPase[1] (EC 3.6.1.3, ATP phosphohydrolase) activity and its digestion with trypsin (Gelotte, 1951; Gergely, 1959; Ellenbogen *et al.*, 1960; Brahms and Kay, 1963; Mueller *et al.*, 1964b; Bárány *et al.*, 1964). Thus, studies on subfragment 1 from heart myosin may help in understanding the relationship of the structure of heart myosin to its ATPase activity.

Bovine heart myosin was chosen because it is more available than rabbit heart myosin. It will be shown in this paper that bovine heart subfragment 1 can be prepared from the papain digestion of bovine heart HMM. This is in contrast to the very small quantities of subfragment 1 which can be obtained from the trypsin treatment of bovine heart HMM.

Materials and Methods

Rabbit skeletal myosin was prepared by procedures previously described (Bárány and Oppenheimer, 1967; Gaetjens *et al.*, 1968).

Bovine heart myosin was prepared as follows: Two hearts were obtained from the local slaughterhouse and fat and extraneous tissue were removed; the muscle then was minced in a precooled grinder. The minced muscle (2000 g) was extracted at 4° with 6.0 l. of a solution containing 0.3 M KCl, 0.075 M KH_2PO_4, 0.075 M K_2HPO_4, and 0.0033 M ATP (pH 6.6). Extraction was continued for 30 min with mechanical stirring. The mixture was centrifuged at 13,000*g* for 15 min and then filtered through a paper pad which had been washed with 0.6 M KCl (pH 7.0). The filtrate (5.8 l.) was diluted with 15 volumes of cold distilled water, and the resulting precipitate was allowed to settle overnight. To remove most of the hemoglobin, the supernatant was decanted and the jars were refilled with cold distilled water and 10 ml of 3.0 M KCl/l. of water was added to maintain the precipitated state of myosin. The washing was repeated at least three times or until the supernatant appeared colorless. The precipitate then was collected and dissolved in 0.6 M KCl in a final volume of 1.5 l. An equal volume of cold distilled water was added, and the pH was adjusted to 6.4–6.5; this suspension was centrifuged at 23,000*g* for 30 min. The supernatant (2.2 l.) was diluted with 10 volumes of cold distilled water. The precipitated myosin was allowed to settle overnight. The myosin was collected and dissolved in 0.6 M KCl at 25 mg/ml. The pH of this solution was adjusted to 6.7 and then clarified in a Spinco preparative ultracentrifuge for 2 hr at 275,000*g* (average force in rotor 50.1). The upper two-thirds of the supernatant was removed as bovine heart myosin. About 14 g of myosin was isolated by

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[1] Abbreviations used are: ATPase, adenosine triphosphate phosphohydrolase; HMM, heavy meromyosin.

this procedure from 2000 g of muscle mince. The myosin was stored in a deep freeze at 13 mg/ml in 50% glycerol before use. No further increase in ATPase activity was observed when this myosin was passed through cellulose phosphate (Gaetjens *et al.*, 1968). The absence of actin from the preparation was indicated by the fact that no ATP sensitivity (Weber and Portzehl, 1952) was found.

Heavy meromyosin from bovine heart or rabbit skeletal muscle was prepared by digestion with trypsin at a ratio of 100:1 by weight (myosin to trypsin) at 25°, pH 6.2, for 30 min (Lowey and Cohen, 1962).

Proteolytic Digestion of Bovine Heart HMM. Bovine heart HMM (10–15 mg/ml) in 0.04 M KCl and 0.001 M Tris-HCl (pH 6.2) was adjusted to the appropriate pH by the addition of 1.0 M Tris-HCl (at various pH values) or 5.0 M sodium acetate (pH 6.2). The digestions were performed at 2 or 25° with trypsin, chymotrypsin, or papain at a ratio of 15:1 by weight (HMM to protease) unless otherwise stated. The pH was maintained by the addition of the appropriate buffer. Trypsin digestion was stopped by the addition of a twofold weight excess of soybean trypsin inhibitor over trypsin; chymotrypsin digestion was stopped by the addition of a 400-fold excess of phenylmethyl sulfonyl fluoride over chymotrypsin (mole/mole); papain digestion was terminated by the addition of a 50-fold excess of iodoacetate over papain (Lowey *et al.*, 1969). After 3 min a twofold excess of cysteine was added to react with excess iodoacetate. The reagents added had no effect on the Ca^{2+}–ATPase activity of bovine heart HMM. For further details, see the legends to the respective figures.

Column Chromatography of a Proteolytic Digest of Bovine Heart HMM. The entire digest of HMM (60–80 mg) was applied to a column (2.6 × 44 cm) of Sephadex G-200 equilibrated with 0.05 M KCl and 0.1 M Tris-HCl (pH 7.6). The column was developed with the same solvent at a flow rate of 10 ml/hr (Young *et al.*, 1965); 3-ml fractions were collected. The recovery of the protein and of the Ca^{2+}–ATPase activity under the major peaks, *i.e.*, peaks I and II, was determined as follows. The protein recovery was calculated as (total $A_{280\ m\mu}$ of the peak/total $A_{280\ m\mu}$ of bovine heart HMM) × 100. The Ca^{2+}–ATPase recovery was calculated as (total Ca^{2+}–ATPase of the peak/total Ca^{2+}–ATPase of bovine heart HMM) × 100. The total Ca^{2+}–ATPase was expressed as micromoles of P_i per minute.

Bovine Heart Subfragment 1. Subfragment 1 was prepared by digestion of bovine heart HMM at 15 mg/ml (50 ml) in 0.04 M KCl with papain at a ratio of 15:1 by weight (HMM to papain) for 40–60 min at pH 7.0, 2°. To stop the reaction, iodoacetate was added as described above. The mixture was centrifuged at 150,000*g* for 60 min to remove papain which had become insoluble under these conditions. F-actin at 10 mg/ml was added to the supernatant at a ratio of 5:1 by weight (original HMM to F-actin) with stirring at 0°. The mixture was centrifuged at 150,000*g* for 2 hr. The supernatant was discarded, and the pellets were homogenized with a solution containing 0.4 M KCl, 0.005 M ATP, 0.005 M sodium pyrophosphate, 0.001 M $MgSO_4$, and 0.004 M Tris-HCl (pH 7.3) to dissociate the subfragment 1 complex of actin (Jones and Perry, 1966). The dissociated complex was centrifuged again in the Spinco at 150,000*g*. The supernatant was then dialyzed at 0° against 0.05 M KCl and 0.0025 M Tris-HCl (pH 7.5), and the solution was changed frequently to remove ATP and pyrophosphate. This solution was concentrated to 7 ml by vacuum dialysis in a collodion bag. The crude subfragment 1, at a concentration of about 15 mg/ml, was purified further by chromatography on Sephadex G-200, as described, and was concentrated by vacuum dialysis. The purified subfragment 1 was dialyzed against 0.05 M KCl and 0.0025 M Tris-HCl (pH 7.5) and then clarified in the Spinco at 150,000*g* for 1 hr.

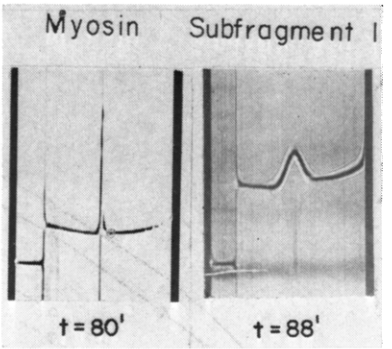

FIGURE 1: Ultracentrifuge patterns of bovine heart myosin and subfragment 1. Myosin: protein concentration, 4 mg/ml; speed, 59,780 rpm; cell width, 12 mm; angle, 65°; sedimentation coefficient, 4.6 S. Subfragment 1: protein concentration, 2.9 mg/ml; speed, 50,740 rpm; cell width, 30 mm; angle, 55°; sedimentation coefficient, 5.5 S In both cases the solvent was 0.5 M KCl and 0.05 M phosphate buffer (pH 7.0) and the temperature was 20°.

Trypsin and soybean trypsin inhibitor and other proteolytic enzymes were obtained from Worthington Biochemical Corp. The trypsin was assayed according to Lowey and Cohen (1962) and its specific activity was 7000 trypsin units/mg. Chymotrypsin was assayed by the procedure of Hummel (1959) and its specific activity was 46.8 units/mg. Papain was obtained as a suspension in 0.05 M sodium acetate and was activated as follows: The suspension (2 ml) was dialyzed exhaustively against 0.005 M cysteine and 0.002 M EDTA (pH 5.9). Under these conditions, the specific activity was 16.7 μmoles of KOH uptake/min per mg when assayed by the procedure of Slutyerman and De Graaf (1969). This papain preparation was used for not longer than 2 weeks.

Rabbit skeletal F-actin was prepared according to a procedure reported earlier (Bailin and Bárány, 1967).

The ATPase activity of myosin and its proteolytic fragments in the presence of Ca^{2+}, at low and high ionic strength, or EDTA was determined as described previously (Bárány *et al.*, 1964).

Amino Acid Analyses. The composition of bovine heart myosin and its proteolytic fragments was determined as described previously (Bárány *et al.*, 1964) with the exception of tryptophan which was determined by the spectrophotometric procedure of Goodwin and Morton (1946). Cysteine was determined as *S*-carboxymethylcysteine after treatment of the proteins with [^{14}C]iodoacetate. To about 15 mg of protein in 0.2 M Tris-HCl (pH 7.4), 125 μmoles of [^{14}C]iodoacetate (0.02 μCi/μmole) was added, and then 1.0 g of solid urea was added per ml of solution. After standing at room temperature for 1 hr, the proteins were precipitated with 10% trichloroacetic acid, washed six times with 2% of this acid and once with 0.1% trichloroacetic acid, and dissolved in 2% NH_4OH. Samples of this solution were taken for protein and radioactivity determinations. The content of carboxymethylated

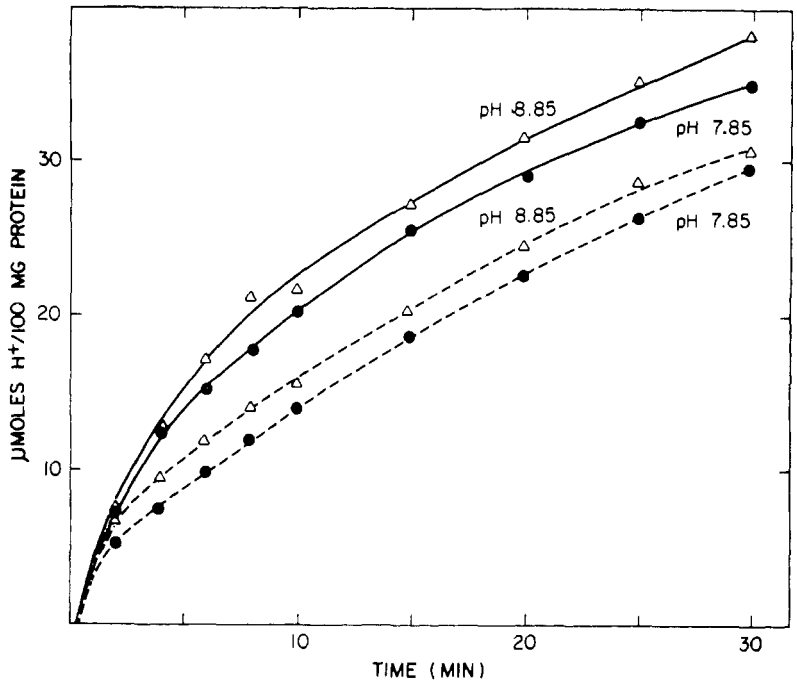

FIGURE 2: Release of hydrogen ions during trypsin digestion of bovine heart and rabbit skeletal myosin at 25°. All studies were performed on 40 mg of myosin in 5.0 ml of 0.6 M KCl, with a myosin to trypsin ratio of 100:1. Solid lines, rabbit skeletal myosin; broken lines, bovine heart myosin. (●) pH 7.85 and (△) pH 8.85.

residues in the proteins was calculated from the known specific activity of the [^{14}C]iodoacetate stock solution. Independent determinations on the amino acid analyzer after acid hydrolysis of the proteins (Bárány *et al.*, 1964) showed that only cysteine residues were carboxymethylated. All *S*-carboxymethylcysteine determinations were performed immediately after the proteins were isolated (Bárány *et al.*, 1964).

pH-Stat Studies. Release of hydrogen ions during proteolysis was followed with a Radiometer pH meter (TTT 1) and Titrigraph (SBR 2). The reaction vessel was placed in a water jacket and the temperature was maintained at 25° or 2.5 ± 0.1°. Exclusion of CO_2 was ensured by passing a stream of prepurified nitrogen gas over the surface of the reaction vessel. Bovine heart and rabbit skeletal HMM in the absence of proteolytic enzymes were tested for spontaneous hydrogen ion liberation, and none was found. The proteolytic enzymes were similarily tested for autolysis. The titrants 0.01, 0.02, and 0.05 N NaOH were standardized with 0.01 N HCl before use with the same pH meter. Unless otherwise stated, all studies were performed at pH 7.85; this represents the p*K* of newly formed α-amino groups as determined by Mihályi and Harrington (1959) for the trypsin digestion of rabbit skeletal myosin. All our values, expressed as μmoles of hydrogen ion per 100 mg of protein, have been so corrected. (The legends to the respective figures give further details.)

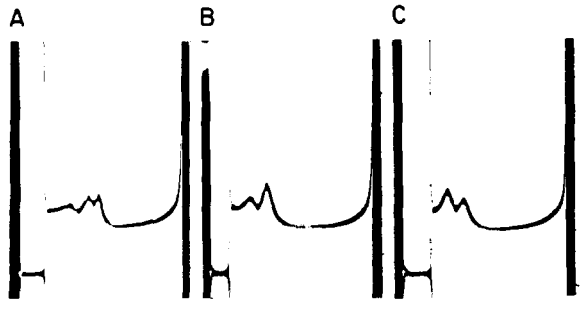

FIGURE 3: Ultracentrifuge patterns of the proteolytic digestion of bovine heart HMM. Direction of sedimentation from left to right. Protein concentration, 6–7 mg/ml; speed, 59,780 rpm; cell width, 12 mm; angle, 55°. (A) Trypsin digestion at 25°, pH 6.2, for 2 hr; Sedimentation coefficients, 3.3, 4.9, and 6.2 S. (B) Chymotrypsin digestion at 2°, pH 7.0, for 3 hr; sedimentation coefficients, 2.4 and 5.4 S. (C) Papain digestion at 2°, pH 7.0, for 2 hr; sedimentation coefficients, 2.7 and 5.3 S. In all cases the ratio of HMM to protease was 15:1. For further details, see Materials and Methods.

Sedimentation Analyses. Sedimentation velocity and equilibrium studies were carried out with a Spinco Model E analytical ultracentrifuge. Schlieren optics equipped with a phase plate were used for the velocity experiments, which were performed in a concentration range of 0.7–6.0 mg of protein/ml, with cells 30–6 mm wide, at speeds of 50,740, or 59,780 rpm, and at a constant temperature of 20°. The Rayleigh interference optical system was used for the equilibrium studies with the three-channel Yphantis cell at 3–8°. The rotor velocity used for the equilibrium experiments of subfragment 1 was 17,980 rpm. The molecular weight of subfragment 1 was calculated according to Yphantis (1964). A value of 0.73 was assumed for the partial specific volume of subfragment 1. The ultracentrifuge plates were read with a Nikon Model 6 optical microcomparator.

Protein was determined by the biuret method of Gornall *et al.* (1949) by measuring the absorbance at 320 mμ (Bailin and Bárány, 1967).

All chemicals were of reagent grade and were used without further purification.

Results

Proteolytic Digestion of Bovine Heart Myosin. This myosin, homogeneous in the analytical ultracentrifuge (Figure 1), was used throughout these studies.

TABLE I: Proteolytic Digestion of Bovine Heart Heavy Meromyosin.

Protease	pH	HMM:Protease (w/w)	Temp (°C)	Time (min)	Protein Recovery (%) Peak I	Peak II	Total[a]	Ca^{2+}-ATPase Recovery (%) Peak I	Peak II	Total[a]
Trypsin	7.0	15:1	25	20	43	22	65	7	11	18
				40	38	19	57	3	7	10
			2	40	71	9	80	53	6	59
				60	69	11	80	51	8	59
				120	58	22	80	45	13	58
	7.6	20:1	25	10	83	17	100	32	4	36
				30	43	17	60	11	15	26
Chymotrypsin	7.0	15:1	2	45	55	28	83	41	32	73
				90	34	42	76	23	39	62
				270	25	43	68	15	31	46
Papain	7.0	15:1	25	10	9	30	39	1	29	30
				20	7	26	33	1	20	21
			2	40	16	48	64	3	68	71
				60	6	53	59	2	62	64
				120	6	51	57	1	61	62

[a] Represents the sum of peaks I and II.

Figure 2 shows that bovine heart myosin was more resistant to trypsin digestion than was rabbit skeletal myosin under identical conditions. This was indicated in both the overall rate and extent of trypsin digestion. For example, in the case of trypsin digestion of bovine heart myosin for 15 min, the release of hydrogen ion per 100 mg of protein was 75% of that of rabbit skeletal myosin at pH 7.85 or 8.85. The differences were even greater at pH 6.85 (not shown). Our data concerning the trypsin digestion of rabbit skeletal myosin are in good agreement with the results of the pH-Stat studies of Mihályi and Harrington (1959).

When bovine heart myosin was digested at pH 6.2 for 30 min at a ratio of 100:1, the corresponding bovine heart HMM was isolated. The analytical ultracentrifuge pattern of bovine heart HMM showed, in addition to the main peak, a slower sedimenting component. This slow component probably was a digestion product or trypsin–trypsin inhibitor complex, or both. When this HMM was chromatographed on Sephadex G-200, the average of five determinations showed that 78% of the protein appeared as bovine heart HMM.[2] No further purification of HMM was undertaken since it was digested with various proteolytic enzymes and the final product, subfragment 1, was purified.

Proteolytic Digestion of Bovine Heart HMM. When bovine heart HMM was digested with trypsin at 25°, pH 6.2, for 2 hr at a 15:1 ratio by weight (HMM to trypsin), splitting of the HMM molecule was observed in the analytical ultracentrifuge (Figure 3A). Sedimentation coefficients of 3.3, 4.9, and 6.2 S indicated the presence of overdigested products or trypsin–trypsin inhibitor complex, or both, subfragment 1, and HMM, respectively. When this tryptic digest was chromatographed on Sephadex G-200, the elution pattern in Figure 4 was obtained A total of 56% protein was recovered in peak I representing undigested HMM or a precursor and 10% in peak II representing a protein resembling subfragment 1. Only 15–16% Ca^{2+}-ATPase activity was recovered in peaks I and II. This was also the case for the EDTA-ATPase activity. No Ca^{2+}-

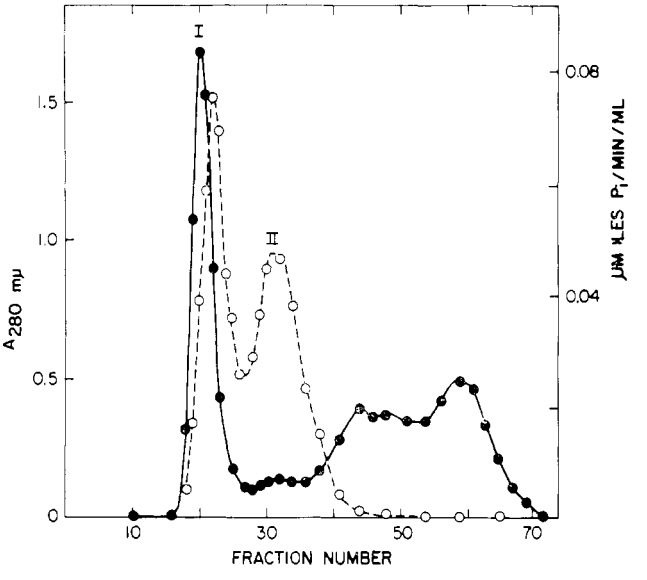

FIGURE 4: Sephadex G-200 chromatography of trypsin-treated bovine heart HMM. The HMM (71 mg) in 0.04 M KCl was digested with trypsin at pH 6.2, 25°, at a ratio of 15:1 (HMM to trypsin) for 2 hr. The final volume of the digest was 5.9 ml before chromatography. For further details, see Materials and Methods. (●) Absorbance at 280 mμ and (○) Ca^{2+}-ATPase activity (micromoles of P_i per minute per milliliter).

[2] This value was used in calculating the recovery of the protein under the peaks in all the chromatograms. Thus, the protein content of bovine heart HMM used for the digestion experiments (expressed as absorbance at 280 mμ) was multiplied by 0.78 to give the actual protein contribution of the HMM.

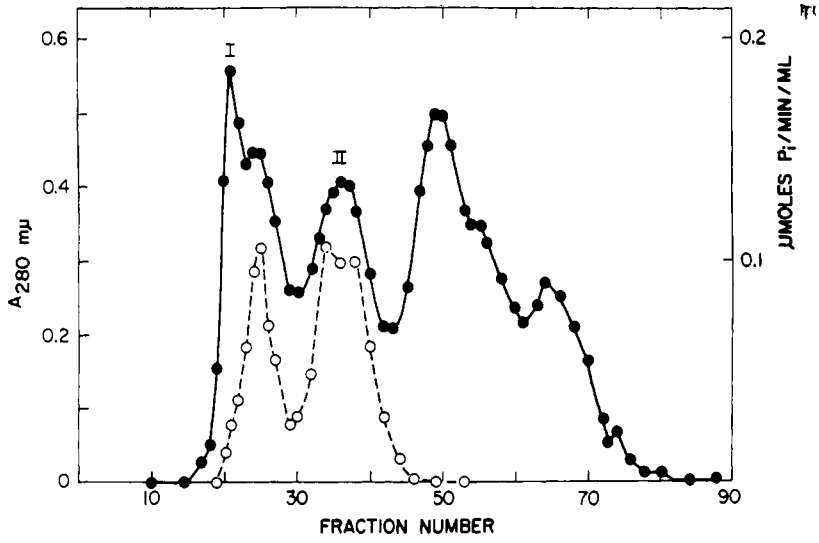

FIGURE 5: Sephadex G-200 chromatography of trypsin-treated bovine heart HMM. The HMM (72 mg) in 0.04 M KCl was digested with trypsin at pH 8.5, 2°, for 2 hr. The final volume of the digest was 3.3 ml, before chromatography. (●) Absorbance at 280 mμ and (○) Ca^{2+}-ATPase activity (micromoles of P_i per minute per milliliter).

or EDTA-ATPase activity was found in the other major peaks which are mixtures of the proteolytic enzyme used and digestion products. When the digestion time was extended, less protein with lower Ca^{2+}- and EDTA-ATPase activities was found in peak II. Thus not much of the protein resembling subfragment 1 was formed.

Table I summarizes the recovery of the protein and Ca^{2+}-ATPase activity of peaks I and II after chromatography of the proteolytic digests on Sephadex G-200. In the case of trypsin digestion at pH 7.6 at 25° and at a ratio of 20:1 the protein resembling subfragment 1 in peak II possessed only slight Ca^{2+}-ATPase activity. In contrast, under identical conditions, subfragment 1 is readily isolated from rabbit skeletal HMM (Mueller and Perry, 1962; Mueller, 1965; Young *et al.*, 1965; Jones and Perry, 1966; Bailin and Bárány, 1968). When the pH was increased to 8.5, at 25°, at a ratio of 40:1 with a digestion time of 20 min, only 12% protein and less Ca^{2+}-ATPase activity were recovered in peak II, whereas 86% protein and 7% Ca^{2+}-ATPase activity were recovered in peak I (not shown in Table I).

In an attempt to maintain the Ca^{2+}- and EDTA-ATPase activities of bovine heart subfragment 1 the temperature was lowered. Trypsin digestion at pH 7.0, at 2°, did not result in an increase of the Ca^{2+}-ATPase activity in peak II (Table I). However, at pH 8.5, the protein resembling subfragment 1 with somewhat higher ATPase activity was formed when the bovine heart HMM was digested with trypsin for 2 hr at 2° at a ratio of 15:1 (Figure 5). In this typical chromatogram, peak II contained 40% protein and 41% of the Ca^{2+}-ATPase activity, and peak I contained 39% protein and 21% Ca^{2+}-ATPase activity. Moreover, at pH 9.0 or 9.5, the same effect was observed. At these high pH values, even though more

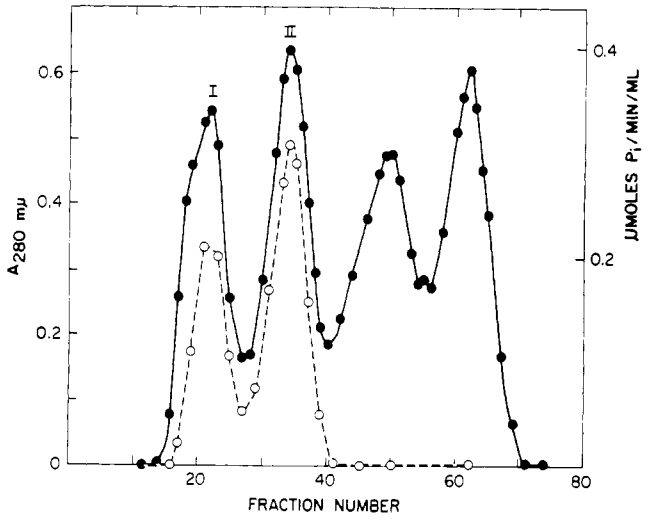

FIGURE 6: Sephadex G-200 chromatography of chymotrypsin-treated bovine heart HMM. The HMM (83 mg) in 0.04 M KCl was digested with chymotrypsin at pH 7.5, 2°, for 1.5 hr. The final volume of the digest was 6.3 ml before chromatography. (●) Absorbance at 280 mμ and (○) Ca^{2+}-ATPase activity (micromoles of P_i per minute per milliliter).

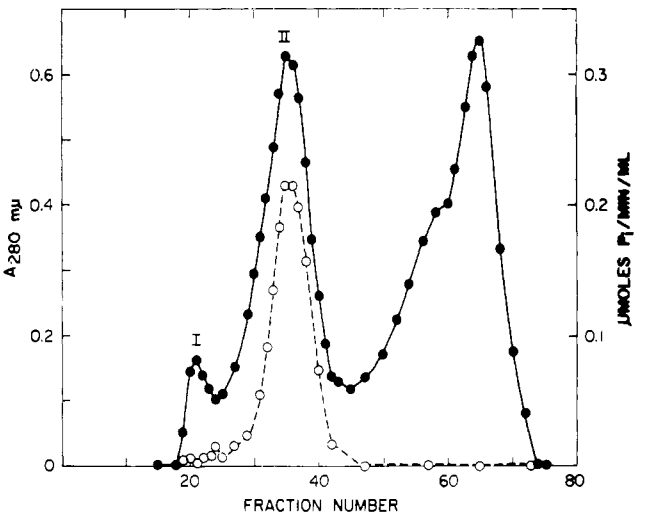

FIGURE 7: Sephadex G-200 chromatography of papain-treated bovine heart HMM. The HMM (72 mg) in 0.04 M KCl was digested with papain at pH 7.0, 2°, for 1 hr. The final volume of the digest was 4.8 ml before chromatography. (●) Absorbance at 280 mμ and (○) Ca^{2+}-ATPase activity (micromoles of P_i per minute per milliliter).

TABLE II: Amino Acid Composition of Bovine Heart Myosin and Subfragment 1.

Amino Acid	Moles/10^5 g of Protein	
	Myosin	Subfragment 1
Cysteine	7.4	9.3
Aspartic acid	92	99
Threonine	44	40
Serine	40	39
Glutamic acid	169	175
Proline	23	25
Glycine	38	44
Alanine	77	72
Valine	38	34
Methionine	23	26
Isoleucine	37	38
Leucine	93	100
Tyrosine	16	17
Phenylalanine	31	33
Lysine	91	90
Histidine	15	17
Arginine	47	42
Tryptophan	3.5	4.6
Total	884.9	904.9

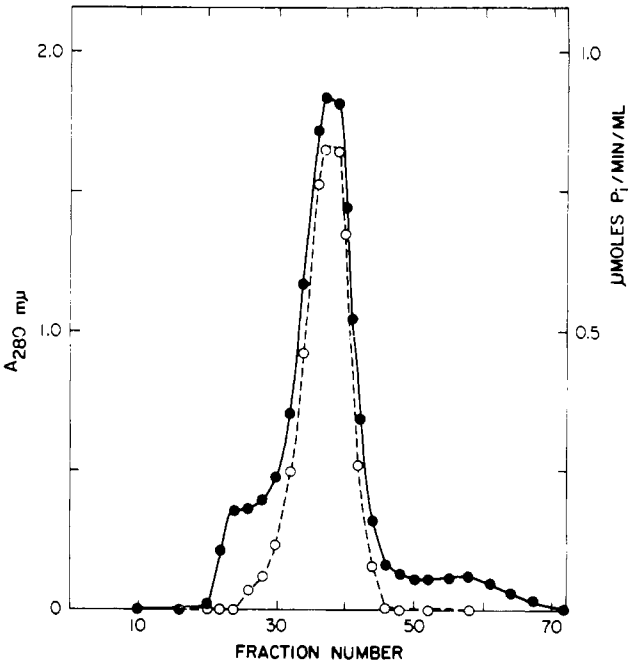

FIGURE 8: Sephadex G-200 chromatography of bovine heart subfragment 1. The subfragment 1 was isolated after actin combination with papain digest of bovine heart HMM and was chromatographed as described under Materials and Methods. (●) Absorbance at 280 mμ and (○) Ca^{2+}–ATPase activity (micromoles of P_i per minute per milliliter).

subfragment 1 appeared in the chromatogram, the ATPase activity was low and the subfragment 1 isolated after actin combination or ammonium sulfate precipitation had very low Ca^{2+}– and EDTA–ATPase activities.

When chymotrypsin was used as the proteolytic enzyme for the digestion of bovine heart HMM, a protein resembling subfragment 1 appeared in the analytical ultracentrifuge (Figure 3B). Sedimentation coefficients of 2.4 and 5.4 S indicated the presence of a small component and subfragment 1. Thus, at pH 7.0, 2°, at a ratio of 15:1 with a digestion time of 90 min and after chromatography on Sephadex G-200, the protein recovery and Ca^{2+}–ATPase activity of peak II were greater than those of peak I (Figure 6). However, subfragment 1 was isolated with low ATPase activity. When the digestion was carried out for 270 min, no significant changes occurred (Table I).

Papain digestion of bovine heart HMM proved to be the most fruitful method of all. Figure 3C represents a typical ultracentrifuge pattern showing two components with sedimentation coefficients of 2.7 and 5.3 S, of which the latter corresponds to subfragment 1. At 25°, pH 7.0, papain digestion of bovine heart HMM for 10 min resulted in 30% protein and 29% Ca^{2+}–ATPase activity in peak II, and 9% protein and 1% Ca^{2+}–ATPase activity in peak I. The HMM apparently was overdigested (see papain samples at 25° in Table I). When the temperature was lowered to 2°, papain digestion of bovine heart HMM resulted in more protein with high ATPase activity recovered in peak II after the mixture was chromatographed (Figure 7). This papain digestion at pH 7.0, for 60 min at a ratio of 15:1, showed 53% protein and 62% Ca^{2+}–ATPase activity in peak II with little protein and Ca^{2+}–ATPase activity in peak I (Table I). Increasing the digestion time to 120 min showed a small increase in the protein content of peak II concomitantly with a decrease in the Ca^{2+}–ATPase activity (see papain samples at 2° in Table I).

For this reason, the papain digestion was terminated after 60 min, and the whole digest was combined with F-actin. After dissociation of the actin complex the crude subfragment 1 was chromatographed on Sephadex G-200. A major peak emerged possessing all the Ca^{2+}–ATPase activity (Figure 8).

Characteristics of Bovine Heart Subfragment 1. The purified subfragment 1 sedimented as a single peak in the analytical ultracentrifuge (Figure 1), and the sedimentation constant was 5.6 S. The plots of the logarithm of the fringe displacement of subfragment 1 *vs.* the square of the radial distance at equilibrium times (Yphantis, 1964) were linear. A molecular weight of 110,000 ± 10,000 was calculated, which is in good agreement with the value obtained for subfragment 1 isolated either from trypsin digestion of rabbit skeletal HMM (Mueller, 1965; Young *et al.*, 1965; Jones and Perry, 1966) or from papain digestion of rabbit skeletal myosin (Nihei and Kay, 1968; Lowey *et al.*, 1969).

The amino acid analyses of bovine heart myosin and subfragment 1 did not reveal significant differences in their amino acid composition (Table II). Heavy meromyosin was analyzed only for cysteine and tryptophan. When compared with rabbit skeletal myosin and its proteolytic fragments, the cysteine and tryptophan content of bovine heart myosin and its proteolytic fragments was lower. For instance, the cysteine content of bovine heart myosin, HMM, and subfragment 1 was: 7.4, 8.2, and 9.3 moles per 10^5 g of protein. These values are less than those of rabbit skeletal myosin, HMM, and subfragment 1, *viz.*, 8.8, 11.0, and 11.0 moles per 10^5 g of protein (Bárány *et*

TABLE III: Effect of pH on the Ca^{2+}–ATPase Activity of Bovine Heart Myosin and Its Proteolytic Fragments.[a]

Protein	pH	μmoles of P_i/min per mg of Protein: Low Ionic Strength	μmoles of P_i/min per mg of Protein: High Ionic Strength
Myosin	6	0.185	0.187
Heavy meromyosin		0.296	0.246
Subfragment 1		0.334	0.305
Myosin	7	0.182	0.169
Heavy meromyosin		0.306	0.210
Subfragment 1		0.497	0.455
Myosin	8	0.190	0.162
Heavy meromyosin		0.348	0.233
Subfragment 1		0.546	0.461
Myosin	9	0.286	0.268
Heavy meromyosin		0.445	0.359
Subfragment 1		0.652	0.635

[a] Reaction conditions: 0.01 M $CaCl_2$, 0.002 M ATP, in 0.03 M KCl (low ionic strength) or 0.25 M KCl (high ionic strength), and 0.02 M Tris-HCl buffer. The assay was started by the addition of the enzyme. The reaction time was 1.5–4 min at 25°.

TABLE IV: Comparison of Papain and Trypsin Digestion of Bovine Heart Heavy Meromyosin.

Temp (°C)	Time (min)	μmoles of H^+/100 mg of Protein: Papain	μmoles of H^+/100 mg of Protein: Trypsin
25	10	39.0	15.0
	20	47.0	21.4
	30	50.4	25.8
	40	54.4	29.8
	50	57.6	33.2
2.5	10	9.7	2.3
	20	12.5	3.0
	30	13.9	3.4
	40	15.1	3.9
	50	16.4	3.9

al., 1964; Jones and Perry, 1966; Lowey *et al.*, 1969). In addition the tryptophan content of bovine heart myosin was 3.5 moles/10^5 g of protein compared with 6.7 moles/10^5 g of protein for rabbit skeletal myosin (Bárány *et al.*, 1964).

Determination of the Ca^{2+}-activated ATPase activities of bovine heart myosin, HMM, and subfragment 1 at various pH values and at high and low ionic strength is shown in Table III. It is clear that bovine heart subfragment I possesses higher Ca^{2+}–ATPase activity at low or high ionic strength than does bovine heart myosin or HMM. Thus, the Ca^{2+}–ATPase activity of bovine heart subfragment 1 was 1.6-fold higher than the HMM and 2.4-fold higher than the original myosin in the pH 6–9 range (Table III).

pH-Stat Studies. Bovine heart HMM was more resistant than rabbit skeletal HMM to proteolytic digestion at 25 or 2.5° (Figures 9 and 10). This is consistent with the observation that bovine heart myosin is more resistant than rabbit skeletal myosin to proteolytic digestion by trypsin (Figure 2). At 25° the digestion of either bovine heart or rabbit skeletal HMM with papain was greater than with chymotrypsin or trypsin (Figure 9). The same effect was observed at 2.5° (Figure 10) although the extent of digestion was very much reduced (*cf.* Figures 9 and 10).

The finding that papain digestion of HMM was greater than chymotrypsin or trypsin digestion was anticipated since papain is a relatively nonspecific protease (Smith and Kimmel, 1960) when compared with chymotrypsin (Desnuelle, 1960) or trypsin (Desnuelle, 1960).

It seemed of interest to correlate the formation of subfragment 1 with the release of hydrogen ions as a result of proteolytic digestion of bovine heart HMM. Table IV shows a comparison of the digestion of bovine heart HMM by papain and trypsin at pH 7.85 and at 25 or 2.5°. On the basis of hydrogen ions released, papain digestion was greater than trypsin digestion at high or low temperature. Results from Sephadex G-200 chromatography of some of these digests indicated that at 25°, after 30 min, at pH 7.85, the protein recovery was 24 and 9% in peak II for the papain and trypsin digestions, respectively. Little Ca^{2+}–ATPase activity in peak II from papain or trypsin digestion was found. At 2.5°, after 30 min, 52% protein in peak II with 71% Ca^{2+}–ATPase activity was recovered in the case of papain digestion, whereas only 10% protein in peak II with 8% Ca^{2+}–ATPase activity was recovered in the case of trypsin digestion.

Discussion

The digestion of rabbit skeletal HMM with trypsin results in the formation of subfragment 1 with high ATPase activity (Mueller and Perry, 1962; Young *et al.*, 1965; Kominz *et al.*, 1965; Jones and Perry, 1966; Lowey *et al.*, 1969). Under identical conditions bovine heart HMM at pH 7.6, 25°, released minimal amounts of subfragment 1 with low ATPase activity. This apparent resistance to proteolytic digestion under various conditions was shown by sedimentation studies (Figure 3A) and by Sephadex G-200 chromatography (Figure 4 and Table I). At the lower temperature of 2° and at various pH values, somewhat larger quantities of subfragment 1 were formed (*e.g.*, see Figure 5), but this subfragment 1 after actin combination or ammonium sulfate precipitation had very low ATPase activity. The reduced tryptic digestion of bovine heart HMM is consistent with the observation that bovine heart myosin was more resistant than rabbit skeletal myosin to trypsin digestion (Figure 2). This has been shown to be the case for other heart myosins (Gelotte, 1951; Gergely, 1959; Ellenbogen *et al.*, 1960; Brahms and Kay, 1963; Mueller *et al.*, 1964b; Bárány *et al.*, 1964).

The digestion of bovine heart HMM by chymotrypsin somewhat altered the pattern observed with trypsin. Sedimentation studies (Figure 3B) indicated the formation of a protein re-

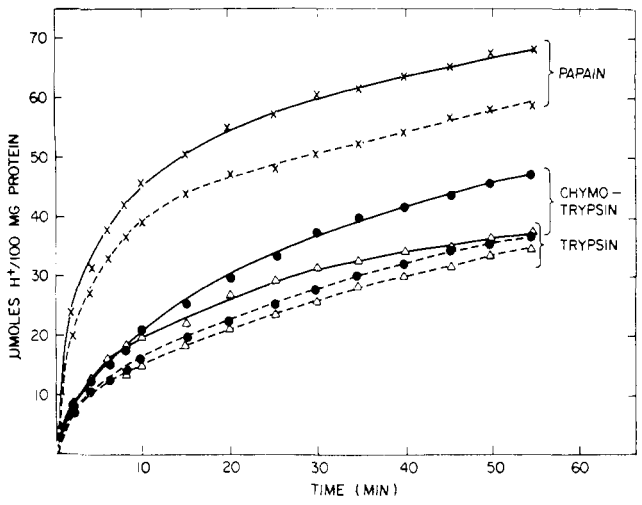

FIGURE 9: Release of hydrogen ions during proteolytic digestion of bovine heart and rabbit skeletal HMM at 25°. All studies were performed on 20 mg of HMM in 0.04 M KCl and 0.001 M Tris-HCl (5.0 ml) with a ratio of HMM to papain, chymotrypsin, or trypsin of 15:1. The release of hydrogen ions was followed as described under Materials and Methods. Solid lines, rabbit skeletal HMM; broken lines, bovine heart HMM. (×) Papain, (●) chymotrypsin, and (△) trypsin.

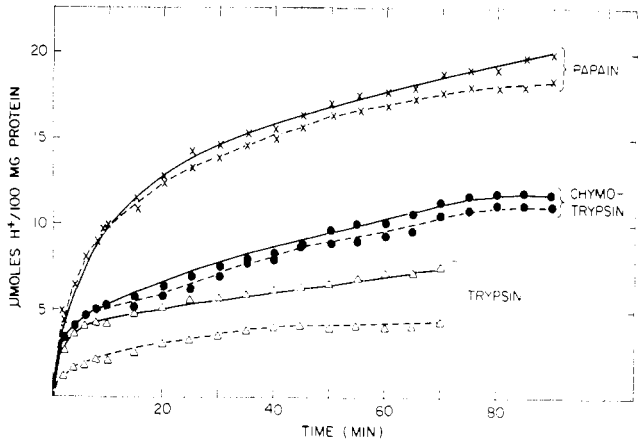

FIGURE 10: Release of hydrogen ions during proteolytic digestion of bovine heart and rabbit skeletal HMM at 2°. In the case of papain and chymotrypsin digestion, 40 mg of HMM in 0.04 M KCl and 0.001 M Tris-HCl in a final volume of 6.0 ml was used. In the case of trypsin digestion, 60 mg of HMM in the same solvent in a final volume of 6.0 ml was used. Under these conditions, the ratio of HMM to protease was 15:1. Solid lines, rabbit skeletal HMM; broken lines, bovine heart HMM. (×) Papain, (●) chymotrypsin, and (△) trypsin.

sembling subfragment 1, and subsequent quantitation of the chymotryptic digest after Sephadex G-200 chromatography showed that more protein with higher Ca^{2+}–ATPase activity under peak II was eluted than in the case of trypsin digestion (compare trypsin and chymotrypsin digestions at pH 7.0, 2°, 40- and 45-min samples, Table I). However, the isolated subfragment 1 (after actin combination or ammonium sulfate precipitation) possessed little ATPase activity.

Papain digestion of bovine heart HMM at 2° did result in the formation of subfragment 1 as shown by sedimentation studies (Figure 3C) and by Sephadex G-200 chromatography of a papain digest (Figure 7). The recovery of the Ca^{2+}–ATPase activity was 68% for the 40-min papain digestion, as compared with 32% for the 45-min chymotrypsin digestion (Table I).

The results of the digestion experiments with papain, chymotrypsin, or trypsin as determined by the pH-Stat give some indication as to the nature of the proteolysis and its relationship to the stability of the ATPase site of bovine heart myosin. At 2.5 or 25°, papain breaks more bonds than trypsin or chymotrypsin in bovine heart HMM (Table IV and Figures 9 and 10). Indeed, more subfragment 1 is formed with high Ca^{2+}-activated ATPase activity in the case of papain digestion at 2° (Figure 7) than in the case of trypsin or chymotrypsin digestion at 2° (Table I, pH 7.0 samples). Although trypsin hydrolyzes fewer peptide bonds than papain (Table IV), apparently trypsin is breaking bonds which are necessary for the maintenance of the active site of bovine heart HMM or subfragment 1 whereas papain is not hydrolyzing such strategic peptide bonds. Therefore, the differences in the action of papain and trypsin on bovine heart HMM are: (i) papain breaks more bonds than trypsin and (ii) papain preferentially splits off the subfragment 1 globules from myosin.

The Ca^{2+}–ATPase activity of bovine heart subfragment 1, although higher than the original myosin (Table III), was much lower than that of rabbit skeletal subfragment 1. Thus, at low ionic strength, *i.e.*, 0.03 M KCl, the Ca^{2+}–ATPase activity (pH 7.0) of rabbit skeletal myosin, HMM, and subfragment 1 (Bailin and Bárány, 1968) was 5.4-, 4.1-, and 4.4-fold higher than the corresponding bovine heart myosin and its proteolytic fragments (Table III). At high ionic strength, *i.e.*, 0.25 M KCl, the Ca^{2+}–ATPase activity of rabbit skeletal myosin and its proteolytic fragments was 3.1- to 4-fold higher than bovine heart myosin and its fragments. The chemical nature of these differences cannot be explained at present. However, they must reflect differences in the ATPase site of rabbit skeletal and bovine heart myosin since the low Ca^{2+}–ATPase activity of bovine heart myosin is also an inherent property of the subfragment 1 isolated from bovine heart HMM.

From our data the stoichiometry of the subunits in bovine heart myosin can be estimated. At present, it appears that rabbit skeletal myosin is composed of two subunits (Slayter and Lowey, 1967; Stracher *et al.*, 1968; Weeds and Hartley, 1968). The results of this paper support the two-subunit concept for bovine heart myosin. Sedimentation studies revealed that the molecular weight of bovine heart subfragment 1 was the same as that of rabbit skeletal subfragment 1 (Mueller, 1965; Young *et al.*, 1965; Lowey *et al.*, 1969). Furthermore, there was no difference in the sedimentation coefficients of bovine heart myosin and HMM when compared with rabbit skeletal myosin and HMM (Mueller, 1965; Young *et al.*, 1965; Lowey *et al.*, 1969). Moreover, the Ca^{2+}–ATPase activity of bovine heart subfragment 1 was 1.6-fold higher than the original HMM and 2.4-fold higher than the original myosin in the pH 6–9 range (Table III). These ratios are in good agreement with the calculated values obtained for two subunits for HMM (350,000/2 × 110,000 = 1.59) and myosin (500,000/2 × 110,000 = 2.28). The results of the chromatography of the papain digestions at pH 7.0 (Table I) and 7.85 showed that 47% of the protein[3] was present as subfragment 1 (Figure 7,

[3] To calculate this value we determined the extinction coefficients at 280 mμ for bovine heart HMM, 0.57 ml mg^{-1} cm^{-1}, and bovine heart

peak II) containing high Ca^{2+}–ATPase activity. Considering the loss of protein which occurs during digestion and column chromatography the 47% value is compatible with the two-subunit concept that 63% of the protein (2 × 110,000/350,000) should be present as subfragment 1.

Despite the similarities in the morphological appearance of heart and skeletal myosin (Mueller *et al.*, 1964a; Carney and Brown, 1966) and in their physical–chemical properties (Gergely, 1959; Mueller *et al.*, 1964b; Luchi *et al.*, 1965) the data of this paper indicate that there are differences in the structure of bovine heart myosin and rabbit skeletal myosin.

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Added in Proof

Dr. Robert Adelstein has found N^{ϵ}-trimethyllysine in both bovine heart myosin and HMM. This amino acid was also observed in rabbit skeletal myosin and HMM (Kuehl and Adelstein, 1969).

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subfragment 1, 0.64 ml mg^{-1} cm^{-1}. These extinction coefficients were used to convert absorbance into milligrams of protein.

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