STUDIES ON SUBFRAGMENT-I, A BIOLOGICALLY ACTIVE FRAGMENT OF MYOSIN*

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The relationship between the structure and function of the myosin molecule is of considerable importance in the understanding of the molecular mechanism of muscle contraction. The nature of the adenosine triphosphatase (ATPase) site and the subunit structure are of special interest in this respect. The hydrolysis of ATP by myosin appears to link the conversion of chemical energy to the mechanical event of muscle contraction.[1] Sulfhydryl groups have long been implicated in this process and two unique sulfhydryl groups have been localized by specific chemical labeling methods.[2-4] Reaction of one of these (S_1) with thiol reagents results in an approximately sixfold increase in Ca^{++}-ATPase,[5] whereas reaction of the second (S_2) results in a loss of Ca^{++}-ATPase.[4] It has been suggested[6] that both of these groups are located directly at the hydrolytic site of the enzyme.

Myosin is composed of subunits that may be dissociated by guanidine or at alkaline pH.[7] The myosin molecule would appear to comprise an axial core of two fibrous (f) or heavy subunits (mol wt 210,000) that terminate in a globular head containing three globular (g) or light subunits (mol wt 20,000).[8-10] Although the S_1 and S_2 sulfhydryl groups are present in the f-chains, the main core exhibits no residual ATPase activity following dissociation of the g-subunits.[10]

Additional information concerning the substructure of myosin has been obtained by means of enzymatic digestion. Myosin may be degraded with trypsin[11] or insoluble papain[12] into a fragment of approximately 120,000 mol wt,[12-17] termed subfragment-I,[11] which contains the full biological activity of the parent molecule. The number of subfragment-I particles produced from myosin has been controversial, with estimates ranging from one to three.[13-15, 17] Recent electron microscopy studies[18] suggest that the myosin molecule has a bipartite head, each lobe of which produces one subfragment-I particle.

This report describes an investigation of the papain- and trypsin-prepared subfragment-I molecules, with particular attention to (1) the presence of the S_1 and S_2 sulfhydryls and (2) the subunit composition of these molecules.

Materials and Methods.—Rabbit skeletal myosin was prepared according to a modification[8, 19] of the method of Szent-Györgyi.[20] The tryptic digestion procedure of Lowey and Cohen[21] was used for the preparation of heavy meromyosin. Actin was extracted and purified according to the method of Carsten and Mommaerts.[22]

The insoluble papain complex (with cellulose) used in the preparation of subfragment-I was made according to the procedure of Lowey.[23] Myosin was digested with the insoluble papain as described by Kominz, Mitchell, Nihei, and Kay.[12] The digestion of heavy meromyosin with trypsin was performed under the conditions described by Mueller and Perry.[11] Subfragment-I was isolated as the F-actin-combining fraction[11] and was further purified by chromatography on Sephadex G-200 (Pharmacia).[14] The protein was concentrated by ultrafiltration under nitrogen pressure. Dissociation studies on subfragment-I were performed after overnight dialysis against 0.4 M KCl–0.1 M Na_2CO_3 at pH 11.0.

Ultracentrifugal studies were performed at 4° in a Beckman model E analytical ultra-centrifuge.[8]   In sedimentation velocity experiments, schlieren areas were corrected for radial dilution; no Johnston-Ogston correction was necessary. High-speed sedimentation equilibrium experiments were carried out by the method of Yphantis.[24] Experiments on subfragment-I at neutral pH were performed at 19,000–24,000 rpm for 30 hr at a concentration of 1.0–2.0 mg/ml. Experiments on subfragment-I at pH 11 were performed at a concentration of 1.5–2.0 mg/ml, reaching successive equilibria at 24,000–26,000 rpm over 36 hr, and 39,000–42,000 rpm over 12 hr. Multicomponent analyses were done by a method previously described[9] in which the molecular weight and concentration of the light component was determined from data at high speed, and the remaining heavy component was characterized from data at high and low speeds after correcting for the presence of light component. The partial specific volume was taken as 0.726 ml/gm for the trypsin-prepared subfragment-I[17] and 0.750 ml/gm for the papain-prepared subfragment-I.[13] A value of 0.726 ml/gm was employed for all fragments isolated at alkaline pH.

For radioactivity measurements, the sample containing less than 4.0 mg protein was pipetted into 15 ml of a dioxane phosphor solution and counted in a Packard Tri-Carb liquid scintillation counter. ATPase measurements were performed according to Perry.[25] Protein concentration was determined by optical density measurement at 280 mμ in a Beckman DU spectrophotometer with correction for Rayleigh scattering. The following values for $A_{280m}^{1\%}$ were used: 5.60[26] (myosin), 6.47[27] (heavy meromyosin), and 7.7[14] (subfragment-I).

1-C^{14}-iodoacetamide (1.1 c/mole) was obtained (in a sealed ampule under nitrogen pressure) from International Chemical and Nuclear Corporation.

Results.—Localization of sulfhydryl groups: The specific labeling of the S_1 sulfhydryl group of myosin is accomplished by reaction with 1.0 mM 1-C^{14}-iodoacetamide for ten minutes at pH 8.3.[28] Blocking of this group leads to a sixfold activation of myosin Ca^{++}-ATPase. After the use of a disulfide-sulfhydryl interchange procedure,[3, 4] the remaining S_2 sulfhydryl is specifically labeled by reaction with 0.1 M 1-C^{14}-iodoacetamide. This modification results in complete loss of ATPase activity.

The distribution of radioactivity in molecular fragments of myosin was determined by the trypsin digestion of the C^{14}-derivatives, which yielded heavy meromyosin and subfragment-I. From 75 to 80 per cent of the specific radioactivity of myosin (molar basis) was found to be associated with heavy meromyosin. Subfragment-I, produced by further tryptic digestion of heavy meromyosin, was purified by chromatography on Sephadex G-200; a typical chromatogram for the C^{14}-S_1-blocked myosin is shown in Figure 1. The conversion of S_1-labeled heavy meromyosin to subfragment-I results in the release of a low-molecular-weight fragment (third radioactive peak) containing over 65 per cent of the initial radioactivity of heavy meromyosin. Subfragment-I (peak 2) retains only 32 per cent of the total radioactivity originally associated with heavy meromyosin but at the same time accounts for more than 90 per cent of the original total Ca^{++}-ATPase. A similar chromatogram was obtained for the subfragment-I that was prepared from the C^{14}-S_2-blocked myosin, this subfragment retaining 31 per cent of the total radioactivity.

Myosin may also be degraded with an insoluble papain complex[12] to produce subfragment-I without the intermediate formation of heavy meromyosin. Subfragment-I was prepared in this manner from S_1-blocked myosin and chromatographed on Sephadex G-200 (Fig. 2). Over 85 per cent of the total radioactivity was found associated with subfragment-I, whereas only a small per-
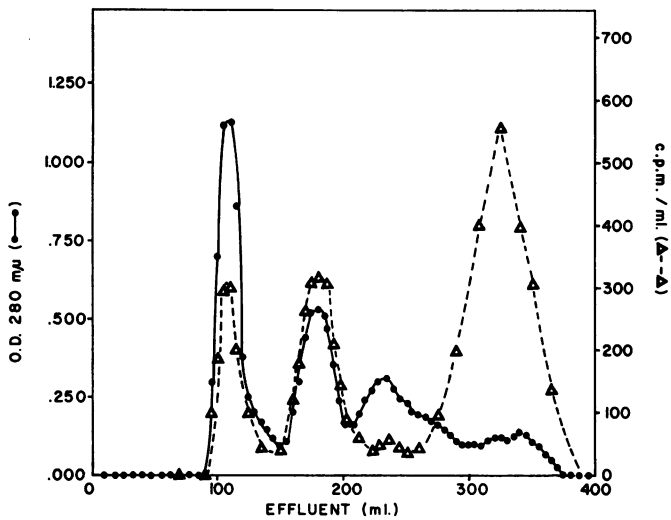
Fig. 1.—Sephadex G-200 chromatography of a tryptic digest of heavy meromyosin prepared from C^{14}-S_1-blocked myosin. To 7.0 ml of a solution containing heavy meromyosin (15 mg/ml; 0.05 M KCl, 0.1 M tris-HCl, pH 7.6; 584 cpm/mg), 0.7 ml containing 5.25 mg trypsin in 0.001 N HCl was added. Reaction proceeded for 25 min at 25° and was terminated by the addition of 10.5 mg soybean trypsin inhibitor dissolved in 0.7 ml water. The entire digest was placed on a 2.5 × 70-cm column of Sephadex G-200 (void vol 110 ml) previously equilibrated with 0.05 M KCl, 0.1 M tris-HCl, pH 7.6, and was eluted with the same buffer at a flow rate of 10 ml/hr at 4°. 5.0-ml fractions were collected and their absorbance at 280 mμ was determined. 2.0-ml aliquots from selected tubes were assayed for radioactivity.

●— —● Optical density at 280 mμ; △- - - -△ radioactivity (cpm/ml).

centage ($<10\%$) was released as a peptide. Results from a similar experiment on the C^{14}-S_2-blocked myosin were essentially the same. On a molar basis, subfragment-I produced in this manner contains 42 per cent of the specific radioactivity of the original S_1-blocked myosin. These data are consistent with the production of two moles of subfragment-I from each mole of myosin. In addition, it can be concluded that tryptic digestion leads to a removal of the S_1 and S_2 sulfhydryls from subfragment-I, whereas papain digestion produces a subfragment-I that retains these residues.

A characteristic feature of myosin Ca^{++}-ATPase is the enhancement of activity by low concentrations of sulfhydryl reagents.[19] Thus the loss of the S_1 sulfhydryl in the trypsin-prepared subfragment-I should diminish the extent of ATPase activation, whereas the papain-prepared subfragment-I should exhibit complete activation. The titration of myosin with 1.0 mM iodoacetamide produces a sixfold activation in the Ca^{++}-ATPase, in contrast to the twofold activation observed for the trypsin-prepared subfragment-I under the same conditions. The papain-prepared subfragment-I is activated fourfold with 1.0 mM iodoacetamide, somewhat less than the maximum activation displayed by myosin. However, when subfragment-I is prepared by digesting S_1-blocked myosin with insoluble papain, the Ca^{++}-ATPase is about six times greater than that found in an unlabeled preparation. This suggests that oxidation of S_1 during the
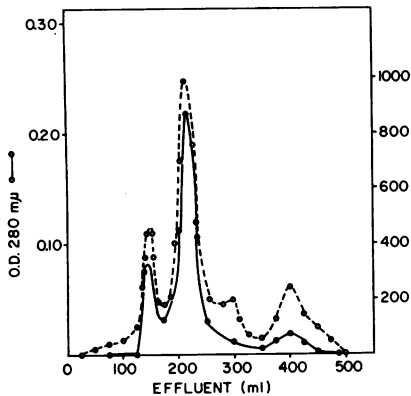
FIG. 2.—Sephadex G-200 chromatography of a papain digest of C^{14}-S_1-blocked myosin. 160 ml of a solution containing myosin (11.9 mg/ml; 0.4 M KCl, 0.05 M KH$_2$PO$_4$/K$_2$HPO$_4$, pH 6.8; 282 cpm/mg) was incubated for 15 min at 25° with the insoluble papain complex containing 9.5 mg enzyme. After removal of the insoluble papain by centrifugation, the supernatant was placed on a 2.5 × 83-cm column of Sephadex G-200 (void vol 140 ml) previously equilibrated with 0.05 M KCl, 0.1 M tris-HCl, pH 7.6. The remainder of the experimental procedure was the same as described in Fig. 1.

O- - -O Optical density at 280 mμ; ●——● radioactivity (cpm/ml).

preparation of subfragment-I leads to the diminished capacity of this particle to be activated to its fullest extent by titration with iodoacetamide.

Ultracentrifugal studies: These experiments were designed to characterize the subunit composition of subfragment-I and the possible structural differences between trypsin and papain preparations. Sedimentation velocity experiments at pH 7.6 on subfragment-I obtained by papain and tryptic digestion indicate $s^{\circ}_{20,w}$ values of 5.5 and 5.8, respectively (Table 1). Sedimentation equilibrium experiments yield average molecular weight values of 108,000 and 104,300, respectively. These values are in agreement with previously reported results.[12–18] Although both preparations aggregated extensively on storage, the trypsin-prepared subfragment-I appeared to be less stable and showed evidence of heterogeneity on sedimentation velocity.

Further studies were carried out after dialysis overnight against 0.4 M KCl, 0.1 M Na$_2$CO$_3$, pH 11.0. On sedimentation velocity, both the trypsin- and papain-prepared subfragment-I demonstrated a dissociation into a light component ($s^{\circ}_{20,w} \sim 1.7$) and a heavy component ($s^{\circ}_{20,w} \smile 5.0$).[29] The percentage of light component was calculated from schlieren area measurements (Table 1) and an average value of 16.0 per cent was found for the papain-prepared subfragment-I. In the case of the trypsin subfragment-I, the trailing component

TABLE 1. *Sedimentation coefficient and fraction per cent of alkali components of subfragment-I from sedimentation velocity.*

		pH 7.6 $s^{\circ}_{20,w}$	Heavy component $s^{\circ}_{20,w}$	pH 11.0 Light component $s^{\circ}_{20,w}$	Per cent*
I.	Papain-prepared subfragment-I	5.5	4.7	1.5	15.9, 18.0
					17.0, 17.2
					14.5, 13.4
	Average (± standard error)				16.0 (±0.72)
II.	Trypsin-prepared subfragment-I	5.8	5.3	1.8	27.0, 28.1, 29.9
	Average				28.3

* For each experiment, the percentages were determined from areas obtained two or three times from 80 to 224 min after reaching 52,640 rpm. Percentages are based on the total area from a synthetic boundary experiment and are corrected for radial dilution.

showed a widely spread, heterogeneous boundary and represented 28 per cent of the total protein.

On sedimentation equilibrium at pH 11, both preparations of subfragment-I showed obvious heterogeneity on graphs of the logarithm of fringe displacement against radial distance squared. The papain-prepared subfragment-I could be resolved into two components (Table 2). The data at 35,000–42,000 rpm indicate a light component of molecular weight 17,900 representing about 13.2 per cent of the total protein. On subtracting the contribution of the light component in each experiment, we found that the remaining heavy component has a molecular weight of about 86,100. The trypsin-prepared subfragment-I displayed greater heterogeneity, with a heavy component having a substantially lower molecular weight of 66,800, a light component of a molecular weight of 18,600, and an additional peptide component of an approximate molecular weight of 2,100. Although the values for the fraction per cent (Table 2) for the two light

TABLE 2. *Molecular weight and fraction per cent of alkali components of subfragment-I from sedimentation equilibrium.*

		pH	Rotor speed (rpm)	Light component		Heavy component	Peptide component	
				Mol wt	Per cent	Mol wt	Mol wt	Per cent
I.	Papain-prepared	11.0	24,630	(17,700)	(14.5)	85,200	—	—
	subfragment-I	"	39,600	17,700	14.5	89,100	—	—
		11.0	24,620	(19,500)	(14.1)	83,800	—	—
		"	42,000	19,500	14.1	—	—	—
		11.0	35,430	17,200	12.8	85,300	—	—
		"	41,820	18,900	12.2	84,600	—	—
		11.0*	24,530	(16,540)	(12.4)	85,520	—	—
		"	39,400	16,540	12.4	89,000	—	—
	Average (± standard error)			17,900 (±500)	13.2 (±46)	86,100 (±1,000)	—	—
II.	Trypsin-prepared	11.0	24,620	(18,600)	(9.2)	63,600	(2,100)	(16.0)
	subfragment-I	"	41,720	18,600	9.2	70,000	2,100	16.0
	Average			18,600	9.2	66,800	2,100	16.0

* Subfragment-I prepared without the use of actin.

components from the trypsin preparation carry considerable uncertainty, the total percentage of the two (25%) is comparable with the value (28.3%) found on sedimentation velocity (Table 1).

A sample of the papain-prepared subfragment-I was carboxymethylated[8] and dialyzed against 5 M guanidine·HCl, 0.4 M KCl, pH 7.0. Sedimentation velocity experiments indicated a main boundary ($s_{20,w}^{\circ}$ = 4S) with an incompletely resolved trailing component. Sedimentation equilibrium at 29,500 rpm and 37,000 rpm indicated a light component of 18,600 molecular weight (20% of the protein) and a heavy component of 79,000 to 87,700 molecular weight.

Studies were also conducted on the effect of papain on the light alkali component of myosin, under conditions identical with those employed for papain digestion of myosin. Sedimentation equilibrium experiments at 40,000 rpm indicate a molecular weight on the order of 13,000–16,000 for the papain-treated g-subunits, a range appreciably less than the 20,000 molecular weight of the

g-subunits of native myosin[9] and somewhat less than the weight of the light component in subfragment-I (Table 2).

C-terminal analysis: Studies on the C-terminal end group of the papain-prepared subfragment-I indicate that carboxypeptidase A liberates approximately one mole of isoleucine from each mole of subfragment-I.   Additional residues were also present but in submolar amounts.

Discussion.—Sulfhydryl groups: In order that the distribution of radiolabel in molecular fragments of myosin reflect the true fate of the S$\cdot$ and S$_2$ sulfhydryls, the initial labeling of myosin must be highly specific for these residues.   Chromatographic separation of radioactive peptides demonstrated[3, 28] that the techniques employed here for chemical labeling of the S$_1$ and S$_2$ groups are in each case highly specific for the labeling of a single, discrete sulfhydryl group per 210,000 mol wt of myosin.   Therefore, the high specific radioactivity of heavy meromyosin (75–80% of that of myosin) can be taken to indicate that the majority of heavy meromyosin molecules retain these two specific groups.   Similarly, the papain-prepared subfragment-I also shows relatively little erosion of the region containing these two functionally important sulfhydryls (Fig. 2).   In contrast, only a minority of the trypsin-prepared subfragment-I molecules retain the radiolabeled sulfhydryls; most of the radioactivity of heavy meromyosin is released as a peptide (Fig. 1).   This result is corroborated in the case of the S$_1$ sulfhydryl by the small (twofold) extent of activation seen for the trypsin-prepared subfragment-I ATPase as compared with the larger change in myosin ATPase (sixfold) and in the papain subfragment-I ATPase (fourfold) upon titration with iodoacetamide.   Thus, since most of the subfragment-I molecules prepared with trypsin have lost the S$_1$ and S$_2$ sulfhydryls that are present in myosin and in the papain subfragment-I, and since subfragment-I prepared by either method retains the full biological activity of the parent molecule, it can be concluded than neither of these sulfhydryl groups is present directly at the active site for ATP hydrolysis.   Rather, it is more likely that these sulfhydryls are located in an allosteric area near the active site where they may influence the catalytic events occurring there indirectly via, for example, a conformational change in protein structure.   As has been suggested,[30] these results seem to imply that there may be a flexible, dynamic character to the ATPase site of myosin, similar to the type of active site associated with allosteric enzymes.[31]

Subunit composition: On alkaline treatment, the papain-prepared subfragment-I is dissociated into a light component (mol wt 17,900) and a heavy component (mol wt 86,100).   The total molecular weight (104,000) is consistent with the molecular weight of subfragment-I (Table 2), and the weight fraction determined from sedimentation equilibrium (13.2%) and sedimentation velocity (16.0%) also suggests the presence of one mole of light component per mole of subfragment-I.   The lack of extensive proteolysis on formation of subfragment-I, as evidenced by the small number of hydrolyzed peptide bonds found in pH stat experiments[13] and the negligible amount of peptide fragments on alkaline treatment of subfragment-I (Table 2), suggests that light and heavy components of the papain-prepared subfragment-I may be derived from subunits of native

myosin.   The heavy component would appear to be a remnant of a single f-chain, while the light component, having a molecular weight (Table 2) significantly less than the 20,000 mol wt of the light alkali component of myosin,[9] may be a partially degraded g-chain.   This conclusion is supported by the presence of a C-terminal isoleucine in the papain-prepared subfragment-I, identical to the C-terminal isoleucine found in the g-subunits,[9, 33] heavy meromyosin,[34] and myosin.[34] The preliminary results on guanidine-treated subfragment-I confirm that the light and heavy components are composed of single polypeptide chains.

Although the trypsin- and papain-prepared subfragment-I molecules have similar molecular weights and similar sedimentation coefficients at neutral pH, there are significant structural differences between these preparations.   On alkaline treatment, the heavy component from the trypsin-prepared subfragment-I has a significantly lower molecular weight (Table 2) and there is present about 12–16 per cent of peptide fragments (mol wt 2,100) not present in the papain preparation.   This is consistent with the observation that the number of hydrolyzed peptide bonds is greater on tryptic[32] than on papain digestion[13] of myosin.

The results are summarized in Figure 3.   The subunit structure of myosin
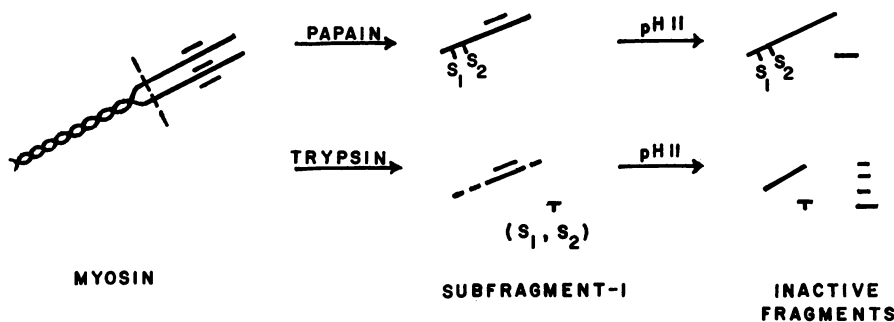

FIG. 3.—Schematic diagram of the subunit structure of myosin and subfragment-I.

has been described previously.[9, 10]   That each mole of myosin produces two moles of subfragment-I was originally suggested on the basis of electron microscopy[18] and is supported by the present data on the specific radioactivity retained in the papain-prepared subfragment-I and also the subunit composition of subfragment-I.   The myosin molecule would thus appear to have a twofold axial symmetry, with each half of the molecule having a biologically active region (subfragment-I) that contains one g-chain and part of an f-chain.   Each f-chain contains the sulfhydryl groups S_1 and S_2 that are implicated in the ATPase activity of myosin.   Their absence from the active site for ATPase suggests that allosteric regulation may be involved in the biological activity of myosin.

Center.   Preliminary reports of this work have been presented at the British Biophysical Society Symposium on Muscle Contraction, London, December 1967, and at the Biophysical Society Meeting, Pittsburgh, February 1968.

‡ Career Scientists of the Health Research Council of the City of New York.

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