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Proteolytic Approach to Structure and Function of Actin Recognition Site in Myosin Heads†

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ABSTRACT: The protective effect of actin against tryptic cleavage of subfragment 1 (S1) heavy chain at the joint connecting the 75K and 20K peptides and against the resulting loss of acto-S1 Mg^{2+}–ATPase activity [Mornet, D., Pantel, P., Audemard, E., & Kassab, R. (1979) *Biochem. Biophys. Res. Commun. 89*, 925] is exercised not only on free S1 but also on the intact myosin. Mg^{2+}ATP and Mg^{2+}ADP impair the protective action of actin to an extent closely related to their respective affinities for the acto-S1 complex. Tryptic fragmentation of S1 heavy chain using trypsin to S1 weight ratios in the range 1:1000–1:1500 indicated that peptide bond cleavage at the 75K–20K joint is a sequential process giving rise first to a 22K peptide intermediate which is subsequently converted into the stable 20K fragment. Most importantly, it is also demonstrated that the loss of S1 activation by actin is not due to the initial scission of the 75K–22K linkage but is intimately associated with the breakdown of the 22K precursor into its 20K moiety. A detailed analysis of the C termini of three trypsin-modified S1 derivatives and of their isolated heavy chain fragments indicated that the 20K fragment is formed mainly through the degradation of an NH_2-terminal 2K segment in the 22K precursor and that this proteolytic event is the only one accounting for the acto-S1 ATPase loss. Cross-linking experiments exploiting the reaction of a carbodiimide reagent with rigor complexes containing either fluorescent actin or fluorescent fragmented S1 revealed unequivocally the attachment of actin monomer to recognition sites on the 20K and 50K units of S1 heavy chain.

The interaction of actin with myosin heads and the actin-dependent activation of the Mg^{2+}–ATPase[1] of the myosin molecule are crucial events of the mechanochemical transduction process in muscle and other motile systems. Whereas the kinetic parameters of the myosin and actomyosin mechanisms have been the subject of extensive research effort in recent years, our knowledge of their structural aspect remains essentially poor. In particular, little of the work on myosin has focussed on the nature and structure of actin recognition

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[1] Abbreviations used: ATPase, adenosine 5′-triphosphatase (EC 3.6.1.3); S1, subfragment 1; acto-S1, actomyosin S1; A1, alkali light chain 1; A2, alkali light chain 2; $NaDodSO_4$, sodium dodecyl sulfate; ATP, adenosine triphosphate; ADP, adenosine diphosphate; PP_i, pyrophosphate; EDC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; HMM, heavy meromyosin; LMM, light meromyosin.

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site in the head moiety (Morales & Botts, 1979); also, no information is available on the location and nature of the peptide components which allow this site to be functionally connected with the ATPase site during the contractile cycle so that force generation at the actin–myosin head interface is always coupled with the chemical steps of the ATPase reaction (Nihei et al., 1974; Marston et al., 1979).

Recently, we have described the primary results we obtained by applying for the first time a biochemical approach which is thought to be potentially efficient in probing the structure and function of the actin site on the heavy chain of rabbit skeletal muscle myosin (Mornet et al., 1979a). This approach is founded on the important property of the intact 95K heavy chain of chymotryptic S1 as well as of papain S1 (Balint et al., 1978) to be rapidly split by mild proteolysis with trypsin into three discrete fragments of M_r 27K, 50K, and 20K which are aligned in this order within the heavy chain polypeptide (Lu et al., 1978) and which remain associated in the fragmented protein; we observed also that concomitantly to the initial cleavage of the chain at the carboxyl joint 75K–20K, the acto-S1 Mg^{2+}–ATPase activity of the enzyme is abolished while all other ATPases remained intact. These results encouraged us to exploit the particular proteolytic sensitivity of the amino 27K–50K and carboxyl 50K–20K junctions for probing the interaction of the heavy chain with actin. When the tryptic digestion is performed with S1 complexed to F-actin, the heavy chain is split only at the NH_2-terminal site giving rise to two fragments of M_r = 27K–70K, and the actin-dependent ATPase activity is completely preserved. Thus, actin appears to modulate both the fragmentation of the heavy chain at the joint 50K–20K and the enzymatic response of S1 to proteolysis at this particular site. We, therefore, postulated that the actin-binding site may be located at or near the heavy chain carboxy-terminal region which extends to the functionally important S1–S2 hinge. Most of our findings have been confirmed by a recent work of Yamamoto & Sekine (1979a,c) conducted along similar lines of investigations.

In the present report we illustrate the specificity of the behavior of F-actin during tryptic digestion of the myosin head, we precise the correlation existing between the loss of acto-S1 Mg^{2+}–ATPase activity and the nature of the proteolytic events occurring at the 50K–20K joint, and, finally, we produce, for the first time, unambiguous chemical evidence for the attachment of actin to the COOH-terminal 20K unit of S1 heavy chain in acto-S1 rigor complexes.

Materials and Methods

Trypsin [treated with L-(tosylamido)-2-phenylethyl chloromethyl ketone] and α-chymotrypsin were purchased from Worthington Biochemical Corp. Carboxypeptidase A (treated with diisopropyl fluorophosphate), carboxypeptidase B, and soybean trypsin inhibitor were obtained from Boehringer Mannheim GmbH, Mannheim, Germany; Constant-boiling HCl was from Merck, Darmstadt, Germany; 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride was from Fluka AG, Switzerland; 1,5-IAEDANS [*N*-(iodoacetyl)-*N'*-(1-sulfo-5-naphthyl)ethylenediamine] was purchased from Pierce Chemical Co.; $NaDodSO_4$ was from Serva, Heidelberg, Germany; Sephacryl S-200, superfine, was from Pharmacia AB, Uppsala, Sweden. All other chemicals were analytical grade.

Myosin was isolated from rabbit back and hind leg muscles according to Offer et al. (1973). S1 was prepared by digestion of myosin filaments with chymotrypsin (Weeds & Taylor, 1975; Wagner & Weeds, 1977), purified as a mixture of S1 isoenzymes by gel filtration over Sephacryl S-200 by eluting with 0.1 M $KHCO_3$, pH 8, and resolved into pure S1A1 and S1A2 isoenzymes by ion-exchange chromatography (Weeds & Taylor, 1975). F-Actin was purified according to Spudich & Watt (1971). The protein pellet was resuspended in 10 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM $CaCl_2$, and 2 mM Tris-HCl, pH 8, containing 1 mM NaN_3 (Taylor & Weeds, 1976). A DNase I assay was used for the determination of the F-actin content of the preparation (Blikstad et al., 1978); this was usually of ~99%.

Protein concentrations were determined by measuring the absorbance at 280 nm with extinction coefficients of $E_{280nm}^{1\%}$ = 5.5 cm^{-1} for myosin (Godgrey & Harrington, 1970), 7.5 cm^{-1} for S1 species (Wagner & Weeds, 1977), and 11.0 cm^{-1} for actin (West et al., 1967). The concentration of proteolytic S1 derivatives was determined according to Lowry et al. (1951) by using bovine serum albumin as the standard. Calculations were based on molecular weights of 472 000 for myosin (Szuchet & Zobel, 1974), 42 000 for actin (Collins & Elzinga, 1975), and 105 000 for S1; the latter value corresponds to a minimum molecular weight determined by analytical ultracentrifugation. The K^+-, Ca^{2+}-, and Mg^{2+}-dependent ATPase activities were measured at 25 °C, pH 7.5, and high ionic strength as specified earlier (Mornet et al., 1979b). For assays in the presence of actin, the medium (1 mL) contained 10 mM KCl, 50 mM Tris-HCl (pH 8), 1.5 mM $MgCl_2$, 5 mM ATP, and 1 mg of actin for 0.05 mg of S1. P_i liberated was measured colorimetrically by an automated phosphate system similar to that described by Terasaki & Brooker (1976). The specific activity of acto-S1 Mg^{2+}–ATPase was 3.2 μmol of P_i min^{-1} (mg of S1)$^{-1}$ while the Mg^{2+}–ATPase of S1 alone was 0.05 μmol of P_i min^{-1} mg^{-1}.

Limited Proteolytic Cleavage of S1 and Acto-S1 Complex. The fragmentation of S1 (2 mg/mL) was carried out with a daily prepared trypsin solution made in 2 mM HCl (1–2 mg/mL); before each set of experiments the specific activity of the protease stock solution was measured with N^{α}-benzoyl-DL-arginine-4-nitroanilide as substrate (sp act. = 1 unit/mg) (Erlanger et al., 1961). The cleavage reaction was performed at various trypsin to S1 ratio (w/w) ranging between 1:100 and 1:1500, in 0.1 M $KHCO_3$ (pH 8) at 25 °C in the interval time 0–30 min in the absence or presence of F-actin; the molar ratio F-actin/S1 was varied between 0 and 2. To follow the time course of changes in enzymatic activity of S1, we removed 25–50-μL aliquots at various times and assayed for ATPase activities; soybean trypsin inhibitor (twice the weight of trypsin present in the sample aliquot) was added to the ATPase assays; no effect of the inhibitor on any ATPase activity was observed. To follow concomitantly the time course of the fragmentation process, we also took 50-μL aliquots in parallel from the digestion mixture and added them to an equal volume of boiling 2% $NaDodSO_4$–5% β-mercaptoethanol solution; after 5 min at 100 °C, samples containing 0.030–0.050 mg of protein were subjected to gel electrophoresis.

The cleavage fragments were separated by electrophoresis in 0.1% $NaDodSO_4$–12.5% polyacrylamide slab gels (Studier, 1973) (1.5 mm thick gels) containing 0.4% bis(acrylamide). A 50 mM Tris–100 mM boric acid buffer (pH 8) was used with bovine serum albumin (68 000), chymotrypsinogen A (25 000), soybean trypsin inhibitor (21 500), myosin light chains (Weeds & Frank, 1972), and rabbit myofibrillar proteins as markers. Gels were stained with Coomassie brillant blue R-250 and destained according to Weber & Osborn (1969).

The preparative isolation of trypsin-modified S1 derivatives is described by Mornet et al. (1981). Heavy-chain tryptic

fragments were isolated with the procedures of Vandekerckhove & Van Montagu (1974) and that of Mornet et al. (1980).

Fluorescent Labeling of Actin and S1 and Protein–Protein Cross-Linking. F-Actin and native S1 were labeled with the fluorescent dye 1.5-IAEDANS essentially as described by Takashi (1979) and by Duke et al. (1976), respectively. The amount of bound dye determined spectrally and by amino acid analysis as *S*-(carboxymethyl)cysteine (Hudson & Weber, 1973) was 0.9 mol/mol of S1 and 0.5 mol/mol of actin monomer. Fluorescent S1 was converted to fluorescent (27K–50K–20K)-S1 derivative by tryptic digestion under the conditions reported above.

Labeled or unlabeled F-actin (3 mg/mL) and labeled or unlabeled fragmented S1 derivative (3 mg/mL) were first combined in 100 mM 2-(*N*-morpholino)ethanesulfonic acid, pH 6 [for experiments performed at pH 7.5, 100 mM *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid buffer was used]; after centrifugation at 160000*g* for 45 min, 4 °C, the pellet, containing at least 95% of the initial amount of protein, was washed twice and resuspended by gentle homogenization in 1 mL of the same buffer. The reagent 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide was then added as a solid to a final concentration ranging between 12.5 and 25 mM, and the reaction mixture was incubated at 20 °C. As controls, F-actin and trypsin-modified S1 were also reacted separately with the carbodiimide reagent. Fluorescent bands were located in the gels by illumination with long-wave ultraviolet light before staining with Coomassie blue.

Amino Acid Analysis. Conventional analyses were carried out in a Beckman analyzer (Model 119B). For C-terminal analyses, digestions with carboxypeptidases A and B were carried out according to Morgan & Henschen (1969).

Results

Specificity of Protection of Myosin Heads by F-Actin against Trypsin-Induced Loss of Actin-Activated Mg^{2+}–ATPase. To evaluate the specificity of the remarkable protective effect of actin against the loss of the actin-dependent ATPase activity of S1 induced by its limited digestion with trypsin, we performed the fragmentation reaction under different experimental conditions. Figure 1A shows the time course of the changes in this enzymatic activity when actin was added incrementally to the digestion mixture. The inhibitory effect of trypsin was progressively reduced and completely disappeared for a molar ratio actin/S1 = 2; a molar ratio of 1 afforded only 50% protection; a stoichiometry greater than 1:1 was expected because the experiments were carried out at ionic strength near 0.1 and it is known that the affinity of S1 for actin is markedly dependent on salt concentration (Margossian & Lowey, 1973; Marston & Weber, 1975). The inhibition of the actin-stimulated ATPase of S1 upon mild trypsin treatment and its total preservation in the presence of a sufficient amount of actin were not unique properties of the free proteolitically produced enzyme. As shown in Figure 1B essentially comparable results were obtained with native myosin. Digestion of the parent protein containing S1 attached as myosin heads was conducted under conditions nearly similar to those used for S1. In 0.1 M $KHCO_3$ buffer, filamentous myosin was even formed allowing the experiments to be performed under rather physiological conditions. Trypsin was added at a protease to myosin weight ratio of 1:100; this ratio, however, was 1:50 relative to the amount of S1 moiety present assuming the mass of the heads to represent about half of the total weight of myosin. By employing an amount of trypsin twice that used for digestion of free S1, we took into consideration the fact that myosin contains trypsin-sensitive sites

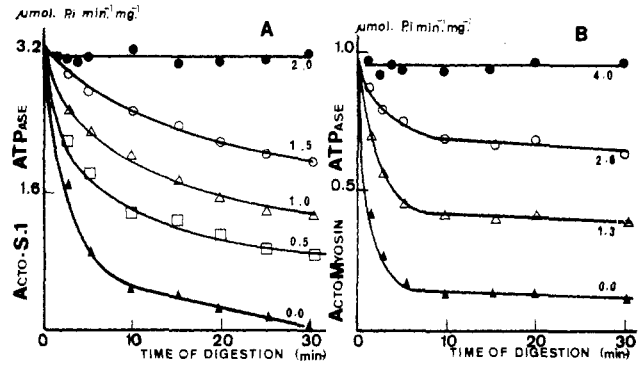

FIGURE 1: Actin protection against trypsin-induced loss of acto-S1 and actomyosin Mg^{2+}–ATPase activity. (A) S1(A1 + A2) (20 μM) was treated with a trypsin to enzyme weight ratio of 1:100 in 100 mM $KHCO_3$, pH 8, 25 °C. The reaction mixture was supplemented with increasing concentrations of actin: 10 μM (□), 20 μM (△), 30 μM (○), 40 μM (●), or no addition (▲); the corresponding actin/S1 molar ratios are reported in the figure. At the times indicated on the abscissa, protein samples were withdrawn and acto-S1 ATPase assays were immediately carried out as described under Material and Methods. The amount of P_i liberated is plotted vs. time of digestion. (B) Myosin (4.5 μM) was incubated with trypsin (protease to enzyme weight ratio 1:100) as described above. Increasing concentrations of actin were added to the digestion medium: 5.9 μM (△), 11.7 μM (○), 18 μM (●), or no addition (▲); the corresponding actin/myosin molar ratios are shown in the figure. Actomyosin ATPase assays were performed on samples withdrawn at the times indicated on the abscissa. The data are plotted as reported in (A).

which are located not only on the S1 moiety but also on the rod portion and which would compete for trypsin (Balint et al., 1975a; Cardinaud, 1979). Under these conditions the time course of loss of the actomyosin ATPase was almost identical with that observed for S1 with $t_{0.5}$ = 2 min. For both S1 and myosin the measured residual enzymatic activity varied between 0 and 8% relative to the control after 25–30 min of digestion. As noted for S1, trypsin did not affect the other ATPase activities of myosin. The protection afforded by actin increased rapidly with increasing actin concentration, and the trypsin effect was abolished at a molar ratio of actin/myosin = 4 which corresponds to a molar ratio of actin/head = 2.

That the disappearance of the inhibitory action of trypsin upon addition of actin is due to the formation of a specific bimolecular complex between actin and S1 or myosin and not due to a bulk viscosity effect or trapping of trypsin by F-actin is evident from three observation. (1) No change in the rate of hydrolysis of N^{α}-benzoyl-DL-arginine-4-nitroanilide (used at 0.1 mM) is noted when the reaction of this synthetic substrate with trypsin is quantitatively investigated in the absence and presence of actin (3.2 mg/mL) under the digestion conditions used for S1. (2) Actin prevents tryptic cleavage at the carboxy-terminal joint 75K–20K of S1 heavy chain but not at the amino-terminal junction 27K–70K (Mornet et al., 1979a); proteolysis at the latter site is, therefore, a direct intrinsic probe of the normal hydrolytic behavior of trypsin in the presence of actin under the conditions employed. (3) The addition of ATP, ADP, or PP_i to digestion mixtures of S1 and actin causes the inhibitory action of trypsin to reappear. There is no question that nucleotides do bind to S1 under the experimental conditions since they cause the dissociation of the S1–actin complex as assessed by separate turbidimetric measurements. As illustrated in Figure 2A, we have followed the time course of changes in the actin-stimulated ATPase of S1 during the tryptic treatment of its complex with actin in the presence of 5 mM Mg^{2+}–ATP and Mg^{2+}–ADP. Upon addition of the nucleoside triphosphate, the protective effect of actin was totally abolished, and the acto-S1 ATPase was

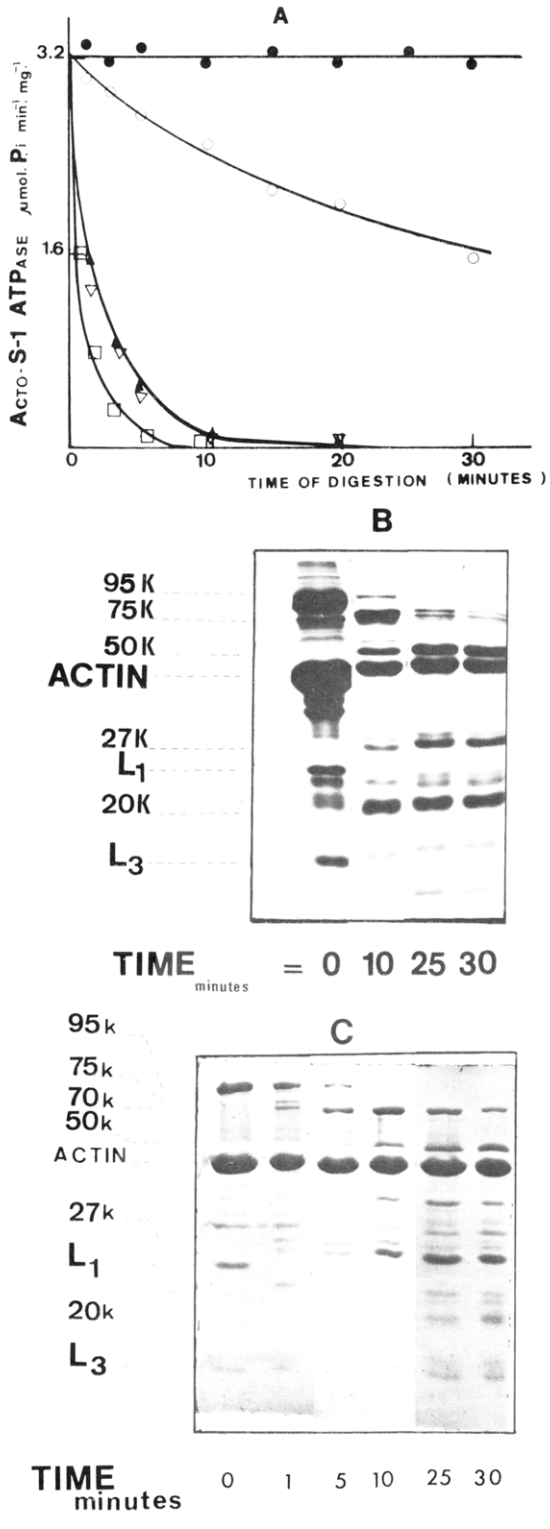

FIGURE 2: (A) Time course of changes in actin-dependent Mg^{2+}–ATPase of S1 during tryptic digestion of the acto-S1 complex in the absence and presence of nucleotides. S1(A1 + A2) (20 μM) was incubated with actin (40 μM) and trypsin (protease to S1 weight ratio 1:100) at pH 8 and 25 °C in the presence of 5 mM Mg^{2+}–ATP (△), 5 mM Mg^{2+}–ADP (○), or no addition (●). Controls are S1 digested alone (▲) and in the presence of 5 mM Mg^{2+}–ATP (□). At the times indicated acto-S1 ATPase activities were measured as described in Figure 1A and under Material and Methods. (B and C) Time course of fragmentation of S1 heavy chain during tryptic digestion of the acto-S1 complex in the presence of Mg^{2+}–ATP and Mg^{2+}ADP, respectively. At the times indicated, protein aliquots were taken from the digestion mixtures described in (A) and sujected to electrophoresis in 0.1% $NaDodSO_4$–12.5% polyacrylamide slab gels with the Tris–borate buffer system as specified under Material and Methods.

lost at a rate identical with that usually observed for S1 alone. During digestion of S1 in absence of actin, no significant influence of ATP on the inactivation reaction was observed, except that, as indicated in Figure 2A, the nucleotide induced occasionally a slight increase of the rate of the acto-S1 ATPase loss. ADP was found to impair but partially the protective effect of actin; after 25–30 min of digestion ~50% of the acto-S1 ATPase remained intact. Thus, the two nucleotide substrates which are known to alter actin binding to S1 (Highsmith, 1976; Beinfeld & Martonosi, 1975) induced also changes in the protection afforded by actin; the extent of these changes appears to be related to the respective affinity of the nucleotides for S1 and to the affinity of actin for the two S1–nucleotide complexes (Marston et al., 1979). The observed distinct effects of ATP and ADP on the time course of the acto-S1 ATPase loss were found to be remarkably reflected in the peptide patterns of S1 heavy-chain fragmentation illustrated in Figure 2B,C. In the presence of actin and ATP, the 95K heavy chain was split into the 75K and 20K peptides followed by the 50K and 27K components. This indicates that proteolysis of S1 has taken place while the enzyme was completely dissociated from actin. In contrast, the banding pattern obtained in the presence of ADP clearly corresponds to the digestion of a mixture of two distinct molecular species, free S1 and acto-S1 complex. This conclusion is consistent with the following: (1) the formation in the early stage of the reaction (1–5 min) of the 75K and 70K fragments which derive from S1 and acto-S1, respectively (Mornet et al., 1979a); (2) the slower rate of appearance of 20K peptide as compared to that observed in the digest containing ATP; (3) the presence of almost equal amounts of 70K and 50K components after 25–30 min of digestion; the former fragment accounts, of course, for the 50% residual acto-S1 ATPase activity measured at the same time of the digestion process (Figure 2A).

Structural Basis of Inactivation of Acto-S1 Mg^{2+}–ATPase by Trypsin. In an attempt to obtain a more precise insight into the relationship existing between the abolition of actin activation of S1 and the structural alterations of its heavy chain in the carboxy-terminal region connecting the 50K and 20K segments, we decided to reinvestigate the tryptic digestion of S1 with the use of relatively low amounts of protease. Figure 3A shows the time course of changes in the actin-stimulated Mg^{2+}–ATPase of S1A1 when proteolysis is effected at a weight ratio of trypsin to enzyme of 1:1000. Over the first 5 min of the reaction period, this activity remained intact; then it decreased progressively reaching 42% of the original value after 20 min. A careful examination of the gel electrophoretic pattern corresponding to this experiment revealed a striking feature of the fragmentation of the heavy chain closely related to the observed loss of acto-S1 ATPase (Figure 3B). During the 20-min period of the reaction, trypsin was acting almost exclusively on the carboxy-terminal side of the heavy chain. The parent 95K polypeptide was partially but significantly split into only two components: the large NH_2-terminal 75K fragment accompanied by a COOH-terminal peptide of M_r = 22K. The formation of the latter material was not observed at all in digests conducted at a higher trypsin to S1 weight ratio; its electrophoretic velocity was slightly higher than that of the 23K breakdown product of the alkali light chain L_1 but slightly lower than that of an authentic sample of 20K fragment used as marker. That the 22K peptide derives from the heavy chain and not from the proteolytically labile L_1 is evident from the observation that it is present in the digests of both S1A1 and S1A2. However, after 5 min of digestion the 22K peptide progressively disappeared with simultaneous appear-

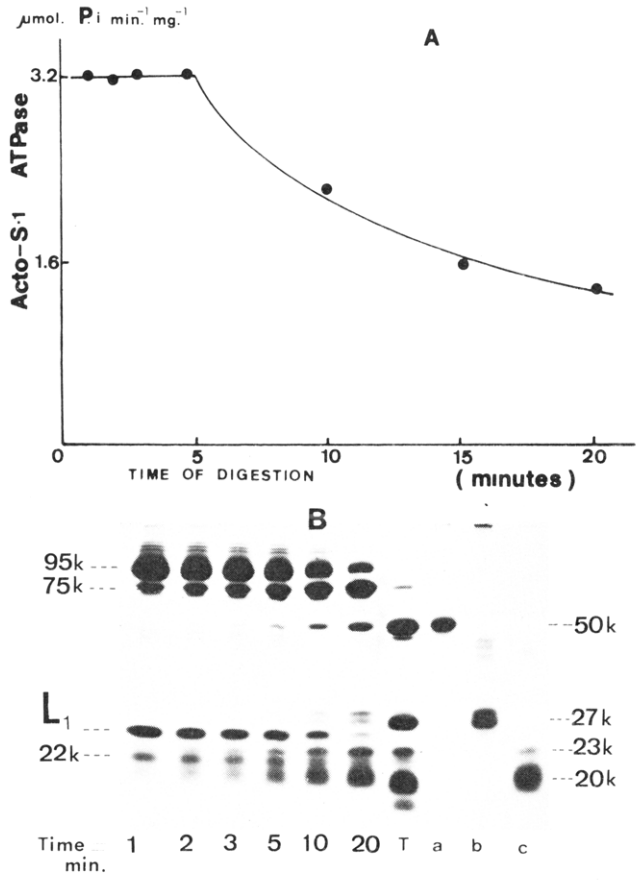

FIGURE 3: Correlation between the loss of actin-stimulated Mg^{2+}–ATPase of S1 and the successive formation of the C-terminal 22K and 20K fragments of the heavy chain. (A) S1A1 (20 μM) was treated with a trypsin to enzyme weight ratio of 1:1000 in 100 mM $KHCO_3$, pH 8 and 25 °C. At the times indicated, samples were taken and assayed for acto-S1 ATPase activity. The data are plotted as reported in Figure 1. (B) Electrophoretic patterns in 0.1% $NaDodSO_4$–12.5% polyacrylamide slab gel of S1 heavy chain after the indicated times of digestion under the conditions depicted in (A). Protein markers are (T), pure (27K–50K–20K)-S1A1 derivative and (a), (b), and (c), pure 50K, 27K, and 20K fragments isolated as described under Material and Methods.

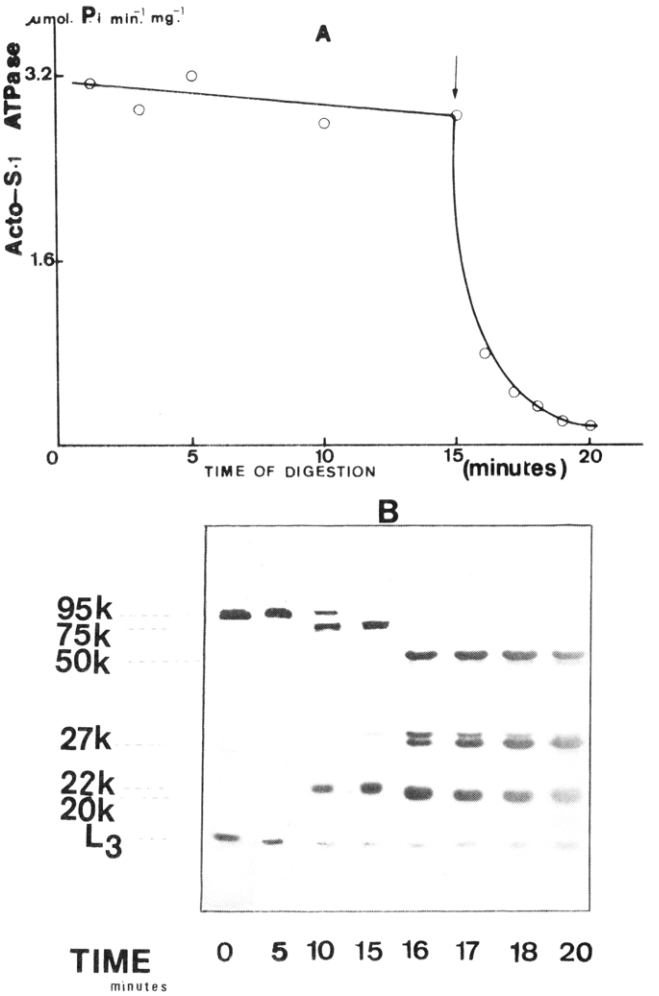

FIGURE 4: (A) Dependence of the acto-S1 Mg^{2+}–ATPase inactivation on the conversion of the 22K precursor into the 20K peptide. S1A2 (20 μM) was incubated with a trypsin to S1 weight ratio of 1:1500 at pH 8 and 25 °C. After a reaction time indicated by the arrow (15 min), this ratio was increased to 1:100 by addition of trypsin. At the times shown on the abscissa, samples were taken and analyzed for actin-dependent ATPase activity. (B) $NaDodSO_4$–12.5% polyacrylamide gel electrophoretic patterns of S1 heavy chain after various times of digestion under the conditions described in (A). Details are given in the text.

ance of the 20K element, while only a very small amount of the NH_2-terminal peptides 27K and 50K was formed. Thus, the 20K peptide is actually issued from a two-step proteolytic process involving first some accumulation of the transient 22K peptide and its subsequent conversion into the stable 20K component. There is little doubt that this pair of fragments deriving from S1 is identical with the two fragments of M_r 23K and 21K identified previously by Balint et al. (1975a), upon limited tryptic hydrolysis of HMM in the absence of divalent cations. The precursor–product relationship between these two peptides was recognized on the basis of their respective comparable rates of appearance and disappearance and also on the basis of the observation that both peptides contain the two reactive SH_1 and SH_2 thiol groups of myosin (Balint et al., 1978). Our own results furnish further the important indication that the rate of extent of conversion of the 22K material into the 20K component account remarkably well for the observed rate and degree of loss of the acto-S1 ATPase. This finding was ascertained by the data obtained with S1A2 under another set of experimental conditions illustrated in Figure 4. A sample of S1A2 was treated with trypsin at a protease to S1 weight ratio of 1:1500. Over a 15-min period of reaction no significant change of the actin-stimulated ATPase was noticed. However, when, at the end of this period, the weight ratio of trypsin to S1 was brought to 1:100 by addition of fresh protease, this enzymatic activity was reduced to ~20% within 1 min and almost completely abolished after 5 min of digestion under these conditions. A comparison of these data with the gel electrophoretic pattern showed that the first phase of digestion performed with a very low amount of trypsin has promoted a slow but complete scission of the initial 95K heavy chain at the 75K–22K junction without alteration of the actin-activated ATPase; in contrast, the second proteolytic phase initiated by an increase of trypsin concentration led to the sharp degradation of the accumulated 22K peptide into its 20K moiety with a concomitant rapid loss of ATPase activity. Of course, there was also an accelerated splitting of the 75K fragment into its 27K and 50K components, but as we have demonstrated earlier (Mornet et al., 1979a) and as illustrated just above, there is no apparent correlation between the formation of these peptides and the modification of the acto-S1 ATPase.

Finally, the study of the gel pattern of Figure 4B has also furnished interesting indications concerning the sequence of proteolytic events giving rise to the NH_2-terminal 27K peptide. This material was frequently observed to originate not from a single peptide bond cleavage on the heavy chain but rather

Table I: Determination of Carboxyl-Terminal Amino Acids in Trypsin-Modified S1 Derivatives and Heavy-Chain Tryptic Fragments by Carboxypeptidases A and B Digestion[a]

S1 derivatives and peptide prepn	carboxypeptidase A		carboxypeptidase B	
	Ile	Phe	Lys	Arg
native S1(A1 + A2)	0.92	0.99	0.25	0
native S1A1	1.00	1.50	0	0
(27K–50K–20K)-S1(A1 + A2)	0.85	0.15	1.95	2.25
(27K–50K–20K)-S1A1			1.54	1.76
(27K–50K–20K)-S1A2			2.10	1.36
(75K–22K)-S1(A1 + A2)	0.88	0.12	0.60	0.80
(75K–22K)-S1A1			0.80	0.70
(75K–22K)-S1A2			0.78	0.85
(27K–70K)-S1(A1 + A2)		0	0.80	1.85
95K heavy chain	0	0.80		
27K fragment			1.00	0
50K fragment			0.75	0
20K fragment			0.15	0.80

[a] All digestion reactions were carried out in 100 mM *N*-ethylmorpholine acetate buffer, pH 8, containing 0.2% $NaDodSO_4$, for 4 h at 40 °C by using protease to substrate weight ratios of 1:30 for carboxypeptidase A and 1:300 for carboxypeptidase B. Values are expressed as moles of amino acid released per mole of S1 or peptide. Values given are the average of two to five C-terminal determinations.

from the degradation of a precursor of M_r = 29K; the 29K material did not accumulate in substantial amount because it was rapidly converted into the stable 27K fragment. Since the latter peptide was reported to contain the blocked NH_2-terminal end of S1 heavy chain (Lu et al., 1978), it is logical to assume that the proteolitically sensitive 2K segment present in the 29K intermediate is located exclusively at the COOH terminus of the peptide. In conclusion, it is worth noting that the amino- and carboxy-terminal 27K and 20K peptides, respectively, of S1 heavy chain were both generated through the breakdown of a specific precursor.

Identification of Sites of Trypsin Cleavage in S1 Heavy Chain. While it was evident that the 22K fragment was issued from a tryptic attack at a protease-sensitive site on the carboxy-terminal segment of the heavy chain, an important question remains to be answered concerning the susceptibility of the remote C-terminal end of the chymotryptic 95K polypeptide to trypsin. This question is particularly relevant to the relationship between the observed loss of acto-S1 ATPase and the parallel conversion of the 22K peptide into the 20K fragment. Indeed, since the former material occupies a C-terminal position in the heavy chain, its breakdown may have occurred according to either of three possible proteolytic pathways: (1) peptide bond cleavage at the NH_2-terminal end; (2) peptide bond cleavage at the C-terminal end; (3) tryptic degradations at both ends of the fragment. In an attempt to determine the exact nature of the hydrolytic events taking place in the heavy chain during the formation of the 22K peptide and its subsequent conversion into the 20K component, we have undertaken a detailed study of the C-terminal structure of the various S1 derivatives and tryptic fragments using carboxypeptidase A and B digestions; for comparison, native S1 was subjected to the same procedures. The results are illustrated in Table I. Upon treatment with carboxypeptidase A, native S1 released stoichiometric amounts of isoleucine and phenylalanine, the other amino acids present being <0.2 mol/mol of S1. Isoleucine is the C-terminal residue of the alkali light chains (Frank & Weeds, 1974); phenylalanine must be at the C-terminal end of the heavy chain since it was also quantitatively released from the dissociated 95K polypeptide; the occurrence of phenylalanine is also in agreement with the well-known substrate specificity of chymotrypsin. Analysis with carboxypeptidase B showed the complete absence of basic end groups in the S1 preparation. The digestion of the (27K–50K–20K)-S1 derivative yielded two important results: (1) the total disappearance of phenylalanine and (2) the release of 2 mol of lysine and 1–2 mol of arginine (the light-chain C-terminal isoleucine was unchanged). The results obtained with the isolated tryptic fragments of the derivative indicated clearly that the two lysines were originated from the 27K and 50K peptides while an arginine residue was at the C terminus of the 20K peptide. The extra 1 mol of arginine occasionally observed in the digest of the whole S1 derivative was due presumably either to a small amount of unspecifically degraded material or to some residual fragments issued from the major sites of tryptic cleavage and which may remain noncovalently attached. The loss of phenylalanine and its replacement by arginine at the C terminus of the C-terminal 20K peptide were a clear indications that trypsin has also attacked the remote C-terminal end of the initial 95K heavy chain. The data obtained from the digestion of the (75K–22K)-S1 derivative provided the additional indication that proteolysis at this site occurs concomitantly with the primary break at the 75K–22K junction, since the 22K precursor peptide also lacked phenylalanine. These findings were expected because the C-terminal segment of chymotryptic S1 heavy chain is actually produced by the limited fragmentation of the flexible, protease-sensitive "swivel" connecting S1 to myosin rod; it is very likely, therefore, that a residual loose end remained still present after the chymotryptic digestion and could be finally eliminated by the subsequent trypsin treatment. Very important, however, is the observation that the degradation of the C-terminal end of the heavy chain has occasioned no effect on the actin-activated ATPase since this enzymatic activity was entirely conserved in the (75K–22K)-S1 derivative. This result was confirmed by the data corresponding to the digestion of the (27K–70K)-S1 derivative with carboxypeptidases A and B. This protein component which forms only upon tryptic hydrolysis of the acto-S1 complex (Mornet et al., 1979a) was also devoid of phenylalanine and contained the expected C-terminal lysine and arginine residues. The proteolysis of the loose C-terminal end of the heavy chain was not prevented by actin binding to S1 and has not induced changes in the actin-dependent ATPase of the enzyme. However, when the (27K–70K)-S1 derivative was resubmitted to a stepwise tryptic treatment similar to that reported under Figure 4 for native S1, it released successively the 22K and 20K peptides with concomitant loss of the actin-activated ATPase. All these data led us to conclude the following. (1) The 70K, 22K, and 20K peptides originated from the truncated C-terminal segment of the chymotryptic heavy chain. The original C-terminal material washed out by trypsin, although unknown, except for phenylalanine, must be of a small mass since its disappearance was not detectable by gel electrophoresis or amino acid analysis of the fragments. (2) The 20K fragment is formed mainly through the breakdown of a NH_2-terminal 2K segment in the 22K precursor. (3) Only the latter proteolytic event is associated with the loss of actin-activated ATPase of S1; this statement is in line with the observation that actin protects only the 50K–20K junction. Finally, the overall action of trypsin on the chymotryptic S1 heavy chain, in the absence and presence of actin, may now be summarized according to the scheme given in Figure 5.

Evidence for Binding of Actin at 50K and 20K Units of S1 Heavy Chain. Since the heavy chain within the (27K–50K–20K)-S1 is a three-fragment complex, this S1 derivative ap-

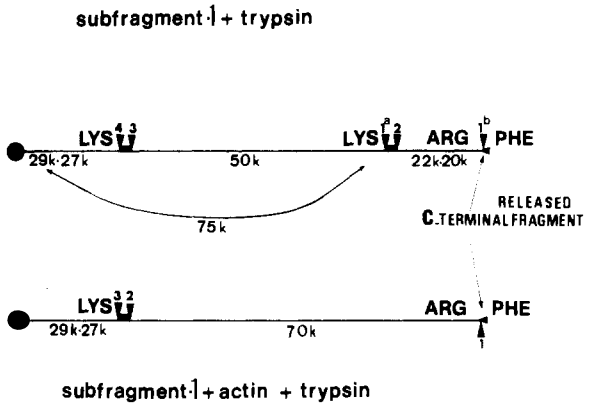

FIGURE 5: Schematic diagram showing the sequence and sites of limited tryptic cleavage on the overall chymotryptic S1 heavy chain in the absence and presence of actin. The arrow heads denote the major sites of tryptic attack which occurs first at the carboxy-terminal sites 1 and 2 and then at the amino-terminal sites 3 and 4. The simultaneous proteolytic events taking place in the C-terminal region during digestion of S1 alone are indicated by (a) and (b) attached to the arrow heads. Actin binding abolishes tryptic hydrolysis at the 50K–20K junction only. For details, see text.

peared as a useful tool for the establishment, by chemical cross-linking experiments, of the nearest-neighbor relationships between actin and the three components of the oligomeric heavy chain. To obtain specific cross-links, we used a water-soluble carbodiimide. This class of reagent has the potent property to promote the covalent cross-linking and polymerization of proteins, particularly in the absence of added nucleophile (Bonner & Pollard, 1975; Timkovich, 1977; Ring & Cole, 1979). It has also the advantage of introducing no new atoms between the groups that it links and thus provides a more stringent test of the proximity of target proteins. However, a serious disadvantage of the carbodiimide reaction is that it yields cross-linked products that are not subject to analysis of protein content by cleavage of the cross-link. But in our case, this difficulty was bypassed and easy recognition of the cross-linked species could be realized by using actin and S1 which, prior to cross-linking, were made fluorescent by a specific reaction with the dye 1,5-IAEDANS at Cys-373 and the SH_1 thiol group, respectively (Takashi, 1979; Takashi et al., 1976). We selected this fluorophore because literature data indicate that it does not appreciably affect the association parameters of the two proteins in the absence of ATP (Nihei et al., 1974; Highsmith et al., 1976; Takashi, 1979). Experiments were also carried out on the complex of the two unlabeled proteins with similar results. Figure 6A illustrates the time course of the reaction of EDC with the complex of actin with trypsin-modified S1(A1 + A2) as analyzed by electrophoresis on an $NaDodSO_4$–10% polyacrylamide slab gel. Over the 15-min period of the reaction, two major new bands were readily formed at an almost identical rate. To the slowest band we assigned an apparent mass of ~92K since its velocity was almost identical with that of S1 heavy chain; the second band showed an electrophoretic mobility slightly higher than that of bovine serum albumin (68K) and was considered as a component of M_r 62K (Figure 6B). When the complex was made with fluorescent actin, the fluorescence was found associated with the two bands; it should be pointed out that no fluorescent protein bands were observed in the 60K–100K region of the gel upon treatment of actin alone with EDC; (a trace amount of 85K actin dimer is observed in overloaded gels.) When the fluorescence in the complex was provided by the labeled S1 derivative, the 62K material appeared fluorescent, together with a faint band of 70K (Figure 6C). Since the label is attached exclusively to the SH_1-containing 20K fragment, these results clearly indicate that the 62K product is an heterodimer of actin (42K) cross-linked to 20K fragment while the 92K component is assumed to be an heterodimer of actin cross-linked to 50K fragment. The use of the S1 derivative containing the fluorescent label at the 20K fragment was essential in proving that the 62K species was not a cross-linked product of actin and an alkali light chain or alternatively of actin and 27K fragment; the actin fluorescence helped also to differentiate the 92K protein band from the intramolecularly cross-linked 97K complex of 50K, 27K, and 20K fragments, the formation of which is reported below. When the cross-linking experiments were conducted at the relatively more physiological pH of 7.5 instead of pH 6, no major changes in the gel patterns were observed except that the amount of cross-linked protein bands was somewhat lower.

We have also investigated the formation of cross-links within the (27K–50K–20K)-S1 when it was treated alone with EDC. The reaction, the time course of which is shown in Figure 6D, yielded two main species of M_r 97K and 70K, the former component being produced in a significantly lower amount. Both material contained fluorescent 20K fragment. They could correspond to covalent complexes of 50K, 20K, and 27K fragments on one hand and of 50K and 20K peptides on the other hand. Comparison of the electrophoretic profiles obtained in the absence and presence of actin (Figure 6C,D) clearly indicates that actin binding to the S1 derivative occasions a dramatic change in the EDC-induced intramolecular cross-linking reaction. In the presence of actin the three fragments-containing 97K product is detectable in trace amount whereas the 50K–20K peptide complex is still formed but in a decreased amount, appearing in the gel as a faint fluorescent band displaying a velocity slightly lower than that corresponding to the dimer of actin with 20K peptide (Figure 6D). In conclusion, the rigor attachment of actin to both 50K and 20K peptides illustrates further the functional role of the 50K–20K junction in S1 heavy chain.

Discussion

A typical structural feature of myosin related to its function is its unusual degree of flexibility which has been first inferred from its proteolytic susceptibility (Lowey et al., 1969). Selective proteolysis of the myosin at two flexible sites has substantiated the existence of the three subfragments or large domains of the protein structure, S1, S2, and LMM. Our previous and present investigations on the tryptic digestion of intact chymotryptic S1 together with the pioneer work of Balint et al. (1975a,b, 1978) on the specific proteolysis of myosin, HMM, and papain S1, provide a further insight into the substructure of the myosin head. The results are compatible with the idea that the 95K heavy chain of S1 is actually a three-domain complex made up of the three main fragments 27K, 50K, and 20K which are convalently connected by two protease-sensitive hinges. A recent ^1H NMR study on myosin and its subfragments has also underlined the unusual high mobility of particular regions located essentially in the S1 moiety of myosin (Highsmith et al., 1979). The three polypeptide units of S1 heavy chain are not released upon the controlled fragmentation of myosin head from fast skeletal muscle myosin only, but at least some of them as well as the intermediate products, 70K or 75K, are also formed in digests of myosin from slow skeletal, cardiac, and embryonic muscle (Balint et al., 1975b); also heavy chain peptide components of M_r 24K and 70K are reported to be present in papain S1 preparations from smooth muscle myosin (Sobieszek & Small, 1976; Marston & Taylor, 1978). Thus it appears to us that

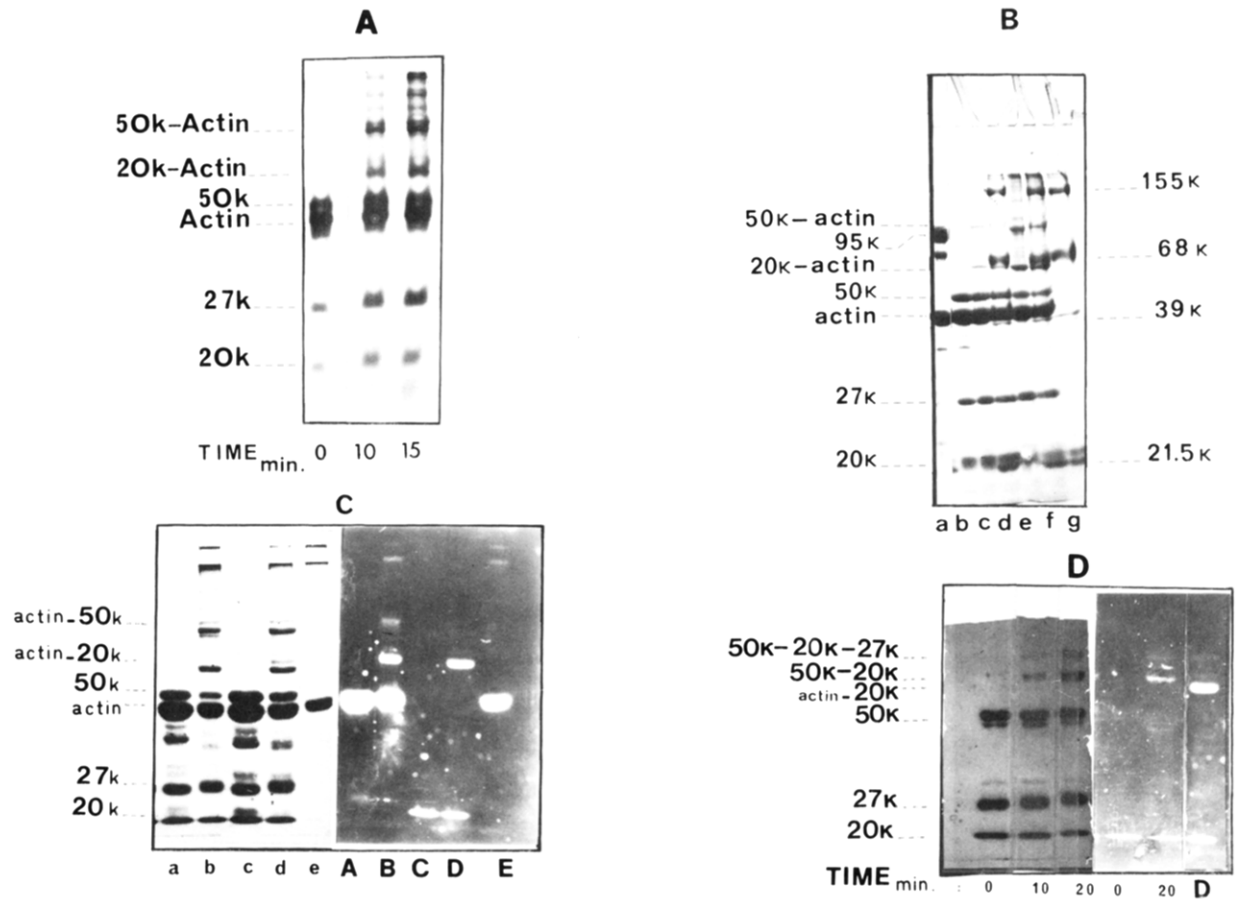

FIGURE 6: (A) Time course of the covalent cross-linking of actin to (27K–50K–20K)-S1 induced by 1-ethyl-3-[3-(dimethylamino)propyl]-carbodiimide. Fragmented S1(A1 + A2) (33 μM) and F-actin (70 μM) were reacted with solid carbodiimide reagent (15 mM) in 100 mM 2-(*N*-morpholino)ethanesulfonic acid, pH 6, 20 °C. At the times indicated, protein samples were analyzed by electrophoresis on 0.1% $NaDodSO_4$–10% polyacrylamide slab gels as specified under Material and Methods. (B) Estimation by gel electrophoresis of the apparent molecular weight of the two major cross-linked protein species formed in the reaction medium described in (A). (a) S1 heavy chain (95K) and actin (42K) run as markers; (b) fragmented S1-actin complex at zero time of the cross-linking reaction; (c and e) protein samples run after 2-min and 10-min treatment with carbodiimide, respectively; (d and f) the same protein aliquots run in the presence of the following protein markers: β and α subunits of *E. coli* RNA polymerase (155K and 39K, respectively); bovine serum albumine (68K); soybean trypsin inhibitor (21.5K); (g) a mixture of the protein markers run separately. Electrophoresis was carried out on 0.1% $NaDodSO_4$–10% polyacrylamide slab gel. (C) Identification of the two cross-linked heterodimers actin–20K peptide and actin–50K peptide. Complexes of fluorescent actin–S1 derivative and of fluorescent S1 derivative–actin were reacted in parallel with carbodiimide as specified in (A); protein samples were submitted to $NaDodSO_4$–10% polyacrylamide gel electrophoresis before (Aa; Cc) and 15 min after addition of the cross-linking agent (Bd; Dd); actin was treated separately with the carbodiimide under similar conditions (Ee). Gels were viewed under ultraviolet light (A–E) and then stained with Coomassie blue (a–e). (D) Time course of carbodiimide-induced intramolecular cross-linking within (27K–50K–20K)-S1; fluorescent fragmented S1 was treated with the cross-linker under the conditions reported in (A); at the time indicated protein samples were subjected to $NaDodSO_4$–10% polyacrylamide slab gel electrophoresis. The fluorescence pattern of the gel following 0- and 20-min reaction time is shown and compared with the fluorescence of the gel corresponding to fluorescent S1 derivative–actin complex treated for 15 min with carbodiimide (D). Note the different mobility of the actin–20K species relative to that of the 50K–20K dimer.

the structure of the whole heavy chain subunit of myosin should be considered as consisting in general not of three but of five folding domains. While the involvement of S2 and LMM, the two COOH-terminal components, in myosin function is well documented, the role of the three NH_2-terminal domains will be, we believe, the matter of a future generation of research efforts which should improve our understanding of the precise molecular mechanism of chemomechanical energy transduction.

In Figure 7 a simple model of myosin head is presented to help us to comment further our results. S1 is considered as a "pear-shaped" protein (Mendelson et al., 1973; Elliot & Offer, 1978), because the mass distribution is highly skewed toward one end. The known linear alignment of the three fragments 27K, 50K, and 20K can be easily fitted into this proposed shape of S1 with the 50K domain constituting at least part of the protein material present in the wide distal portion. The controlled tryptic conversion of monomeric 95K heavy chain into a three-subunit complex represents a helpful approach to the study and localization of the critical binding interactions between the heavy chain and actin as well as between the heavy and light chains by applying procedures currently used for oligomeric proteins such as the chemical cross-linking experiments described in the present report. It can be expected also that hybridization experiments between tryptically fragmented S1 from different myosins would provide valuable information on the degree of complementarity between the three heavy chain domains.

The 50K unit is joined to the 27K and 20K components by two flexible interdomain connector segments of M_r 2K, the presence of which is clearly demonstrated through the progressive breakdown of the 29K and 22K precursors with a low amount of trypsin. In both segments the initial trypsin-sensitive sites, if not most of the basic sites cleaved by the protease, are mainly lysyl bonds; this conclusion is consistent not only with the presence of C-terminal lysine in the 27K and 50K fragments but also with our observation that the two segments are completely insensitive to treatment of S1 with mouse

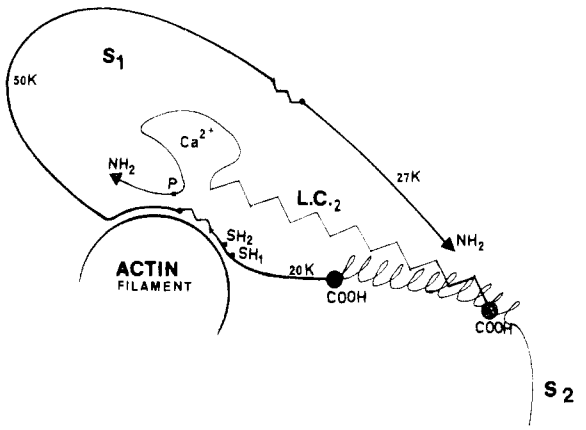

FIGURE 7: Myosin head model proposed to illustrate the three-domain structure of the heavy chain, the interaction of actin at or near the 50K–20K joint and the possible regulatory association of the LC_2 light chain with the 20K domain. The LC_2 subunit is shown as a globular and elongated protein able to span a great part of the head (Alexis & Gratzer, 1978) with its C-terminal half serving for the conservative recognition of the 20K–S2 junction (Kendrick-Jones & Jakes, 1976). SH_1–SH_2 groups are positioned in the NH_2-terminal region of the 20K unit according to Gallagher & Elzinga (1980). For details, see Discussion.

submaxillary gland protease, the specificity of which is highly restricted to arginyl residues (Boesman et al., 1976). While the 27K–50K junction appears outside the acto-S1 interface, the COOH-terminal segment joining the 50K and 20K peptides is of particular interest as far as it is essential for the expression of the acto-S1 function. The ^1H NMR measurements of Highsmith et al. (1979) have indicated the existence in chymotryptic S1 of a 150-residue region showing a remarkable mobile structure; actin binding to S1 specifically abolishes this motile character. The mobile area is assumed to be likely present in that part of the "swivel" between S1 and S2 which may remain on the proteolytically prepared S1 as well as in the actin binding site. Our results can provide further and more precise information concerning the location of the mobile portion of S1. First, there is apparently only little peptide material with loose structure issued from the S1–S2 junction and remaining at the C-terminal end of chymotryptic S1 heavy chain. Secondly, actin binding is not found to affect significantly the tryptic susceptibility and therefore the flexibility of this residual material; conversely, the tryptic degradation of the C-terminal end of S1 heavy chain has no appreciable effect on the acto-S1 function as illustrated by the ability of (27K–70K)-S1 and (75K–22K)-S1 derivatives to be fully activated by actin and to bear a truncated C-terminal segment. Together, our results are in agreement with the fact that, to our knowledge, there is no report suggesting that actin may affect the structure of the S1–S2 junction in the absence of ATP; the only protein ligand known to alter the flexibility of this region is the LC_2 light-chain subunit (Weeds & Pope, 1977); also, it must be remembered that actomyosin preparations are commonly used for the proteolytic isolation of S1 (Cooke, 1972). Consequently, it is logical to conclude that the region of S1, the mobility of which is specifically quenched by actin, resides quite entirely in or near the actin binding site and that the 50K–20K joint we have probed is, most probably, part of this motile structure. The attachment of actin to the 20K domain as evidenced by our cross-linking experiments provides the attractive possibility that this C-terminal segment of the heavy chain is also an important contributor to the observed internal motions of S1.

The flexible 50K–20K junction appears functionally silent in the absence of actin but operant in its presence. This particular area of the heavy chain does not seem to be part of the ATPase site since its proteolysis has no influence on the ATPase activities of S1 alone. Owing to its location at or near the actin-S1 interface and to the high degree of flexibility of its structure, the 50K–20K linkage may play a major role in mediating critical functional interactions between 50K and 20K domains during the different steps of the association of the head with actin. The preparation of the (75K–22K)-S1 derivative makes it possible to isolate the 22K fragment; knowledge of the primary structure of its NH_2-terminal part may provide further insight into the precise role of the 50K–20K joint.

Protein–protein protection effects against proteolysis have been used previously to probe the interaction of LC_2 light chain with the region of the heavy chain at the S1–S2 junction. We have also exploited the dramatic change in the tryptic sensitivity of S1 upon binding to actin to gain information about the structure of the actin recognition site on the heavy chain. Using the zero-length carbodiimide reagent as cross-linker, we obtained direct evidence of the proximity and therefore possible attachment of actin to the 20K C-terminal domain within rigor complexes of F-actin bound to the (27K–50K–20K)-S1 derivative. Yamamoto & Sekine (1979b), using dimethyl suberimidate as cross-linker, have considered the possible formation of a cross-linked product of actin with 50K fragment. Surprisingly, no attention was paid to an eventual cross-linking of actin to 20K peptide in spite of the concomitant occurrence of another cross-linked species of mass in the vicinity of 70K. Our result is of particular interest for the following reasons. (1) The 20K peptide contains the pair of reactive SH_1–SH_2 thiol groups, the region around which is presumed to be part of the ATPase site or to communicate with it (Elzinga & Collins, 1977; Shukla et al., 1979). The binding of actin to a specific area of the 20K unit rationalizes a great body of evidence indicating that ligation of actin to S1 strongly changes the chemical reactivity of SH_1 and SH_2 as well as the environment of chemical probes attached to them (Barany & Barany, 1972; Schaub & Watterson, 1972; Kameyama & Sekine, 1973; Duke et al., 1976; Seidel, 1973); also the reported cooperative effect of actin and ATP binding to S1 on the conformational changes induced by both ligands in this region (Kameyama et al., 1977; Kameyama, 1980) is rationalized. Thus, for the first time, the 20K unit provides a serious structural element through which the actin and ATPase sites may be coupled. (2) The interaction of actin with myosin head can be modulated by a myosin-linked regulation mechanism involving a calcium binding and phosphorylatable light chain subunit; this important control process is thought to be mediated by specific interactions between this light chain and the heavy chains (Szent-Györgyi, 1975). Kuwayama & Yagi (1977) provided evidence that in pig cardiac myosin the attachment of this light chain to the head involves a 5K peptide stretch which extends the C-terminal end of the usual chymotryptic 95K heavy chain. Also, in the case of rabbit skeletal myosin, Hozumi et al. (1979) observed the direct binding of the LC_2 light chain to chymotryptic S1A1 itself which bears a 95K heavy chain. The ability of the 20K domain to associate with actin, on one hand, and to lie close to that part of the S1–S2 junction serving for the permanent attachment of the light chain, on the other hand, encourages us to propose for the first time that the LC_2 subunit and 20K segment represent a potential interacting system contributing to the molecular mechanism of myosin-linked regulation of actomyosin interaction; this system may offer a structural basis for regulation through Ca^{2+} binding in molluscan myosin and

through phosphorylation–dephosphorylation in smooth muscle and cytoplasmic myosins. Our proposal is supported by different findings relevant to the LC_2 light-chain function. Mg^{2+} binding to LC_2 light chain of skeletal myosin profoundly increases the chemical reactivity of SH_1 thiol group (Watterson et al., 1979); conversly, the blocking of SH_1 and SH_2 in this myosin abolishes Ca^{2+} binding to the light chain (Srivastava & Wikman-Coffelt, 1980); chemical modification of SH_1 in gizzard myosin mimics the physiological phosphorylation of the regulatory light chain and induces the activation of the Mg^{2+}–ATPase by actin (Seidel 1979); proteolytic removal of LC_2 light chain from cardiac myosin enhances actin–myosin interaction but also causes changes in the ATPase activities which are similar to those due to SH_2 modification but which are reversed upon recombination of the light chain (Malhotra et al., 1979). In this set of observations the SH_1 and SH_2 thiols present in the 20K fragment behave as sensitive probes of the proposed interaction of the 20K domain with the LC_2 light chain. The 20K segment is also interacting with the 50K unit as evidenced by their chemical intramolecular cross-linking and is, of course, covalently related to the S1–S2 hinge where molecular movements occur during the contractile cycle (Huxley & Simmons, 1971). All these properties strongly suggest that the entire 22K element is a key piece in the structure and function of the myosin head.

Finally, the occurrence of acto-50K and acto-20K cross-linked species does not necessarily mean that the actin subunit contacts the heavy-chain fragments of a single S1. Since we did not observe a cross-linked product containing one actin bound simultaneously to 50K and 20K peptides, the possibility must be considered that cross-linking between the heavy-chain fragments of one S1 and at least two actin monomers might take place. Work is under way to precise the mode of attachment of 50K and 20K domains to the actin filament.

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Comparison of the Binding of Heavy Meromyosin and Myosin Subfragment 1 to F-Actin†

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ABSTRACT: The binding of heavy meromyosin (HMM) to F-actin was examined at varying ionic strengths and temperatures and in the presence of ADP and AMPPNP and then compared to the binding of subfragment 1 (S-1) under identical conditions. In the absence of nucleotide (μ = 0.12–0.43 M, 22 °C), HMM binds 100–1000-fold more strongly to F-actin than does S-1. This indicates that, in the absence of nucleotide, both heads of HMM bind to F-actin, with the second head making a significant contribution to the free energy of binding. On the other hand, in the presence of ADP (μ = 0.43 M, 22 °C) or AMPPNP (μ = 0.12 M, 22 °C), the binding of HMM to F-actin is quite similar to the binding of S-1, indicating that here the second head of HMM does not make a strong contribution to the free energy of binding. In fact, in the presence of AMPPNP, HMM appears to bind to F-actin primarily with one head, while the detached head may be interfering with the binding of another HMM molecule at an adjacent actin site. With all of the different agents tested (ionic strength, temperature, and nucleotide), the effect of the agent on the binding of HMM to F-actin is approximately the square of its effect on the binding of S-1 to F-actin, results consistent with these various agents affecting the binding of *each* of the two HMM heads to the same extent as they affect the binding of an S-1 head.

The myosin molecule has a structure consisting of a long rodlike portion with two globular heads at one end (Lowey et al., 1969). The rodlike portion causes the myosin molecule to aggregate at low ionic strength and is thus integral to the formation of the thick filament. Each globular head has the ability to bind actin and hydrolyze ATP (Lowey & Luck, 1969; Nauss et al., 1969; Eisenberg & Moos, 1970; Eisenberg et al., 1972; Margossian & Lowey, 1973). The myosin molecule can be degraded by proteolysis to produce two soluble fragments, heavy meromyosin (HMM),[1] a double-headed fragment, and subfragment 1 (S-1), a single-headed fragment. Since HMM is two headed and is therefore usually considered a model for myosin, it is important to examine the binding of HMM to F-actin under different conditions.

In the absence of nucleotide, HMM binds to F-actin significantly stronger than S-1, although there is controversy over the magnitude of the actin–HMM association constant. Greene & Eisenberg (1980a) previously found that the association constant for the binding of HMM to F-actin is 3×10^9 M^{-1} at μ = 0.22 M, 22 °C, while under the same conditions, the actin–S-1 association constant is 5×10^6 M^{-1}.

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[1] Abbreviations used: S-1, subfragment 1; HMM, heavy meromyosin; acto-S-1, a complex of actin with S-1; acto-HMM, a complex of actin with HMM; DTT, dithiothreitol.