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Tryptic Cleavage and Substructure of Bovine Cardiac Myosin Subfragment 1†

Dianne Applegate, Armando Azarcon, and Emil Reisler*

ABSTRACT: The method of limited tryptic proteolysis has been used to compare and contrast the substructure of bovine cardiac myosin subfragment 1 (S-1) to that of skeletal myosin S-1. While tryptic cleavage of cardiac S-1, like that of skeletal S-1, yields three fragments, the 25K, 50K, and 20K peptides, the digestion of cardiac S-1 proceeds at a 2-fold faster rate. The increased rate of cleavage is due entirely to an order of magnitude faster rate of cleavage at the 25K/50K junction of cardiac S-1 compared to that of skeletal, with approximately equal rates of cleavage at the 50K/20K junctions. Actin inhibits the tryptic attack at this latter junction, but its effect is an order of magnitude smaller for the cardiac than for the skeletal S-1. Furthermore, the tryptic susceptibility of the 50K/20K junction of cardiac S-1 in the acto–S-1 complex is increased in the presence of 2 mM MgADP. This effect is not due to partial dissociation of the cardiac acto–S-1 complex by MgADP. Our results indicate that in analogy to skeletal S-1, the cardiac myosin head is organized into three protease-resistant fragments connected by open linker peptides. However, the much faster rate of tryptic cleavage of the 25K/50K junction and also the greater accessibility of the 50K/20K junction in the cardiac acto–S-1 complex indicate substructural differences between cardiac and skeletal S-1.

Investigations of the properties of cardiac myosin often involve the application of techniques previously used to characterize the skeletal protein. The rationale of such studies on cardiac myosin is not only to further characterize this protein but also to compare and contrast its respective properties to those of skeletal myosin in an effort to find major determinants of the differing contractile properties of the two types of muscle.

For example, the enzymatic characterization of cardiac myosin has proceeded along such lines. Steady-state measurements indicate that the rate of MgATP hydrolysis by bovine cardiac myosin subfragment 1 (S-1)[1] at saturating concentrations of actin and ATP, V_{max}, is approximately 5-fold less than that of rabbit skeletal S-1 (Taylor & Weeds, 1976). Since the kinetic mechanism for the hydrolysis of ATP in the presence and absence of actin appears to be the same for both skeletal and cardiac subfragment 1 (S-1), the respective rate and equilibrium constants for the various steps in the kinetic scheme can be directly compared (Taylor & Weeds, 1975; Flamig & Cusanovich, 1983; Siemankowski & White, 1984). Such comparisons may have implications for the molecular mechanism of both skeletal and cardiac muscle contraction.

Likewise, the recent derivation from constructed clones of the amino acid sequence of portions of two rabbit cardiac myosin isozymes (Kavinsky et al., 1984) has allowed their direct comparison with the sequence obtained for rabbit skeletal myosin (Capony & Elzinga, 1981). Although the partial sequences of both clones exhibit extensive homology to the analogous regions of rabbit skeletal myosin, specific regions show a particularly high frequency of nonconservative substitutions. While these results are intriguing, the functional

† From the Department of Chemistry and Biochemistry and the Molecular Biology Institute, University of California, Los Angeles, California 90024. *Received May 8, 1984.* This work was supported by U.S. Public Health Service Grant AM 22031, the Muscular Dystrophy Association of America, the Laubisch Fund, and an American Heart Association postdoctoral fellowship to D.A.

[1] Abbreviations: S-1, myosin subfragment 1; PMSF, phenylmethanesulfonyl fluoride; Ap_5A, P^1,P^5-di(adenosine-5′) pentaphosphate; Tris, tris(hydroxymethyl)aminomethane; SDS, sodium dodecyl sulfate; LC-1, light chain 1.

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significance of such substitutions is still a matter of speculation.

Other approches which proved successful in characterizing rabbit skeletal myosin have in general been less extensively applied to cardiac myosin. One such approach which has only been seldom applied to cardiac myosin is the modification of specific residues on the myosin head [e.g., modification of the SH_1 thiol of cardiac myosin (Klotz et al., 1976) or modification of the reactive lysine on cardiac myosin (Oplatka et al., 1976)] and observation of the consequent changes at either the actin or the nucleotide binding sites.

Similarly, while the recent use of tryptic proteolysis of rabbit skeletal S-1 has led to important progress in the investigation of the substructure of this protein, its application to cardiac myosin has only been of a preliminary nature (Flink & Morkin, 1982; Korner et al., 1983). In the work to be described, we have used limited proteolysis to compare and contrast the substructure of bovine cardiac myosin S-1 to that of skeletal S-1. The 95K heavy chain of rabbit skeletal S-1 is cleaved by trypsin to produce three discrete fragments, the 25K, 50K, and 20K peptides. We confirm the preliminary observations that tryptic cleavage of bovine cardiac S-1 produces the same three fragments (Flink & Morkin, 1982; Korner et al., 1983). Furthermore, we show that the peptide linker region constituting the 25K/50K junction in cardiac S-1 is much more susceptible to trypsin than the analogous region of skeletal S-1, while the 50K/20K junctions of the two proteins are equally susceptible. Despite the equivalent rates of cleavage at this latter junction, we also show that actin has much less of a protective effect on its cleavage in cardiac S-1 than in skeletal S-1.

Materials and Methods

Chemicals. Trypsin (crystallized, bovine XI), soybean trypsin inhibitor, α-chymotrypsin, catalase, pepstatin A, and phenylmethanesulfonyl fluoride were purchased from Sigma Chemical Co. (St. Louis, MO). All other reagents were reagent grade.

Proteins. Skeletal myosin and actin from back and leg muscles of rabbits were prepared according to Godfrey & Harrington (1970) and Spudich & Watt (1971), respectively. Cardiac myosin from bovine ventricular muscles was prepared according to Leger et al. (1975). Rabbit skeletal and bovine cardiac myosin subfragments 1 were prepared by digestion of myosin filaments with α-chymotrypsin (Weeds & Pope, 1977). A number of previous studies on cardiac myosin S-1 have show it to be less stable than the rabbit skeletal myosin S-1 (Taylor & Weeds, 1976; Flamig & Cusanovich, 1983). Accordingly, we used only freshly prepared protein in all experiments. The rate of actin-activated MgATP hydrolysis of our preparations ($V_{max} \sim 5.0\ s^{-1}$) agreed well with that quoted by Siemankowski & White (1984). The activity measurements were done as before (Applegate & Reisler, 1983), at 25 °C in a medium containing 5 mM KCl, 25 mM Tris, 3 mM MgATP, pH 7.6, S-1 (0.1 mg/mL), and actin at concentrations ranging from 10.0 to 200 μM.

Proteolysis. In all digestions, the reaction mixture included 0.1 M NaCl and 20 mM imidazole, pH 7.0, with 1.5–2.0 mg/mL final S-1 concentrations. Reaction mixtures were held at 19–20 °C. Digestions were started with the addition of trypsin to S-1 at the weight ratio of 1:400, respectively. In digestions of acto–S-1, the molar ratio of actin to S-1 was 4:1. To follow the proteolytic reaction, aliquots were removed and added to soybean trypsin inhibitor (3× enzyme concentration) to stop the reaction at specific times. The samples were denatured and run on SDS–polyacrylamide gels. For quantitation purposes, catalase was added as an internal standard,

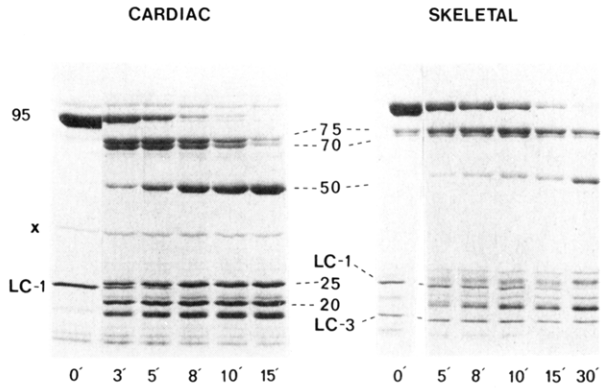

FIGURE 1: Representative electrophoretic pattern of comparative tryptic digestion of bovine cardiac and rabbit skeletal myosin S-1. The molecular size of the fragments is given in kilodaltons between the panels. Skeletal light chains LC-1 and LC-3 and bovine cardiac myosin light chain 1 (LC-1) are indicated. Digestion times in minutes are indicated below each lane. Other reaction conditions were as described under Materials and Methods. It may be noted that an unidentified contaminant band was sometimes observed in our cardiac myosin S-1 preparations (labeled x). This band did not comigrate with actin, and its presence did not affect either the ATPase activity or the tryptic cleavage pattern of cardiac S-1.

in most cases. Gel electrophoresis was carried out according to Laemmli (1970) using discontinuous (10% and 15%) (w/v) polyacrylamide gels. The optical densities of Coomassie blue stained protein bands and the respective mass distributions were determined with a Helena (Beaumont, TX) Quick R&D gel scanner equipped with an integrator. The molecular size of fragments was estimated by comparing their electrophoretic mobilities to that of marker proteins.

Digestion rates were obtained by monitoring the decay of the respective bands as a function of reaction time. All decay curves were fitted by single exponentials and yielded the first-order rate constants for cleavage of S-1 (Applegate & Reisler, 1984). In order to examine the time course of production of the various fragments, we quantitated their formation relative to the extent of heavy chain digestion; i.e., the normalized amount of each fragment formed at a given time was divided by the theoretical maximum amount of this fragment that could be produced from the cleaved fraction of the heavy chain.

Binding Experiments. The binding of S-1 to actin in the presence and absence of ADP was determined by quantitating, on SDS–polyacrylamide gels, the concentration of free S-1 after sedimentation of the acto–S-1 complex ± nucleotide in a Beckman air-driven ultracentrifuge. Using procedures similar to those described by Chalovich & Eisenberg (1982), we centrifuged fractions of 0.17 mL in a Beckman Airfuge at 180000*g* for 30 min. The final concentrations of S-1 and actin, prior to sedimentation, were those of the digestion experiments (i.e., 2 and 3.1 mg/mL, respectively). After sedimentation, 100-μL aliquots were removed, denatured, and subjected to gel electrophoresis. Quantitation was performed as described above.

Results

Comparative Digestions of Cardiac and Skeletal S-1. Flink & Morkin (1982) and Korner et al. (1983) have noted that tryptic cleavage of bovine cardiac S-1 produces the same three fragments, the 25K, 50K, and 20K fragments, as previously observed for skeletal S-1 (Balint et al., 1978). Figure 1 confirms these preliminary observations and shows that under the same reaction conditions the digestion of cardiac S-1 proceeds at a faster rate than that of skeletal S-1. Monitoring

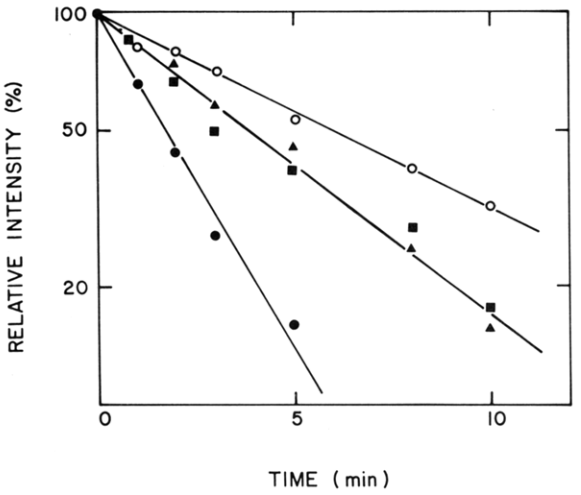

FIGURE 2: Tryptic degradation of the 95K heavy chain of skeletal myosin S-1 (○) and of bovine cardiac myosin S-1 (●). Relative intensities of the 95K heavy chain band are plotted vs. digestion time. To measure the rate of tryptic cleavage at the 25K/50K and the 50K/20K junctions in bovine cardiac myosin S-1, the combined intensities of the 95K and 75K (■) and of the 95K and 70K (▲) bands are plotted vs. time of digestion. The common decay curve indicates that $k_1 = k_2$.

the disappearance of the 95K heavy chain vs. time of digestion reveals that the overall rate of cleavage is 2-fold faster for the cardiac S-1 than for the skeletal S-1 (Figure 2).

Further examination of the representative electrophoretic pattern of the tryptic digestion of cardiac S-1 in Figure 1 shows that both the 20K and 25K fragments are present at the first time point of digestion and, moreover, that the two peptides appear to be produced simultaneously. The tryptic cleavage of the 95K heavy chain can be described by two separate sequential reactions:

$$95K \xrightarrow{k_1} 70K + 25K \quad (1a)$$

$$70K \xrightarrow{k_2} 50K + 20K \quad (1b)$$

$$95K \xrightarrow{k_2} 75K + 20K \quad (2a)$$

$$75K \xrightarrow{k_1} 50K + 25K \quad (2b)$$

Solution of the kinetic rate equations reveals that the rate of cleavage at the 25K/50K junction, k_1, can be determined by monitoring the disappearance of the combined intensities of the 95K and 75K peptides. Likewise, the rate of cleavage at the 50K/20K junction, k_2, can be determined by monitoring the disappearance of the combined intensities of the 95K and the 70K peptides. Figure 2 shows such an analysis of the digestion reaction. Clearly, the cleavage rates at the 50K/20K and the 25K/50K junctions are approximately equal for cardiac S-1. This is in contrast to skeletal S-1, where, as documented by Muhlrad & Hozumi (1982), k_2 is an order of magnitude greater than k_1. Remarkably, however, the k_2 rates are about the same for cardiac and skeletal S-1 (Table I).

In the case of skeletal myosin S-1, cleavage of the 50K/20K junction is a sequential process giving rise first to the 22K intermediate which is subsequently converted to the 20K peptide (Mornet et al., 1981). Likewise, the 25K peptide is generated via 29.5K and 27K peptides (Muhlrad & Hozumi, 1982). Doublets are also apparent for both the 25K and the 20K fragments in the tryptic cleavage of cardiac myosin S-1 (Figure 1). Thus, it is likely that these peptides are generated via precursors as well. It may also be noted that the degradation of the cardiac LC-1 is significantly faster than that of the skeletal LC-1 (Figure 1 and Table I).

Table I: First-Order Rates for Tryptic Proteolysis of Cardiac and Skeletal S-1[a]

protein	rate (min^{-1})			
	k	k_1	k_2	k_{lc}
cardiac S-1	0.37	0.18	0.185	0.45
skeletal S-1	0.17	0.01	0.17	0.18
cardiac acto–S-1	0.24	0.18	0.02	1.4

[a] k_1 and k_2 are the rates of cleavage at the 25K/50K and the 50K/20K junctions, respectively. k is the overall rate of heavy chain degradation obtained from semilogarithmic plots of the 95K intensity vs. time, and k_{lc} is the rate of light chain degradation. The cleavage of skeletal acto–S-1 was too slow to be accurately measured under the employed experimental conditions. All rates are an average of three separate determinations. The experimental variation in these determination was ±10%. Other experimental details are given under Materials and Methods.

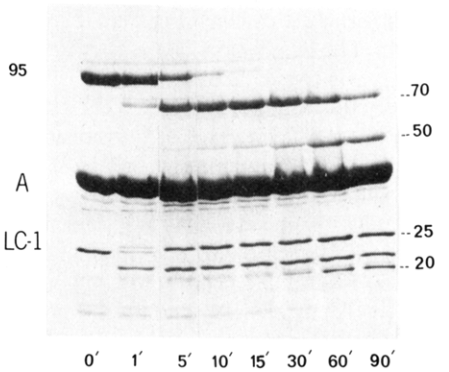

FIGURE 3: Effect of actin on the tryptic digestion of bovine cardiac myosin S-1. The numbers next to the bands are in kilodaltons. Actin is denoted as "A", and LC-1 is indicated.

Effect of Actin on the Tryptic Cleavage of Cardiac S-1. Two actin binding sites have been identified on skeletal myosin S-1, one located on the 20K and the other on the 50K peptide. The first indication that the 50K/20K junction of skeletal S-1 is intimately involved in the actin binding to S-1 was provided by Mornet et al. (1979) and Yamamoto & Sekine (1979), who demonstrated that a 4:1 molar ratio of actin to S-1 effectively blocks the proteolysis of the 50K/20K junction in the skeletal protein. We thus wished to examine the effect of actin binding on the tryptic digestion of cardiac S-1. As can be seen in the electrophoretogram shown in Figure 3, a 4:1 molar ratio of actin to cardiac S-1 inhibits but does not block the cleavage of the 50K/20K junction. With time, there is a significant accumulation of the 50K and the 20K peptides in tryptic digestions of the cardiac acto–S-1 complex. Under identical reaction conditions, the skeletal S-1 does not yield any 50K and 20K peptides. Determination of the rates of tryptic cleavage reveals that the binding of actin to cardiac S-1 causes an order of magnitude decrease in k_2 and no change in k_1 (Table I). This is to be compared to at least 2 orders of magnitude actin-induced decrease of k_2 in skeletal S-1. Thus, the protection afforded this junction by actin is less effective for cardiac than for skeletal S-1.

Further examination of the electrophoretogram shown in Figure 3 reveals another feature of the effect of actin on the tryptic cleavage of cardiac S-1. The degradation of LC-1 is accelerated 3-fold, as monitored by the disappearance of this band vs. time of digestion (Table I).

No measurable changes in either the rates or the cleavage pattern are found upon addition of 5 mM MgATP or 2 mM MgADP to the tryptic cardiac S-1 digestion medium. This is in contrast to the nucleotide-induced changes in the cleavage pattern reported for skeletal S-1 (Muhlrad & Hozumi, 1982).

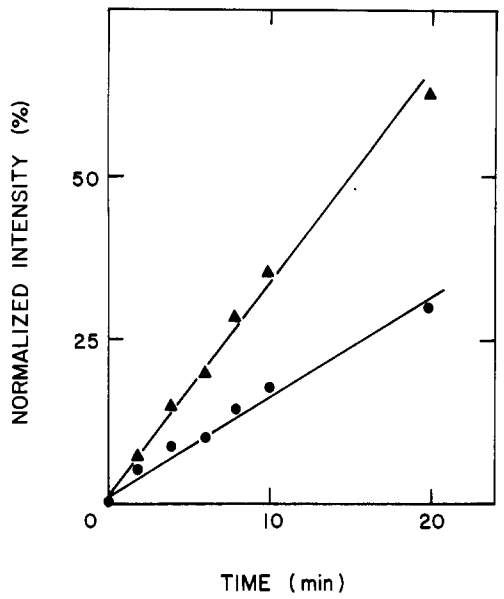

FIGURE 4: Effect of addition of 2 mM MgADP on the production of the 50K fragment in tryptic digestions of bovine cardiac acto–S-1 complex. Relative amounts of the 50K peptide obtained in the absence (●) and presence (▲) of 2 mM MgADP were calculated as described under Materials and Methods.

However, addition of 2 mM MgADP to cardiac acto–S-1 significantly increases the cleavage at the 50K/20K junction. This is shown in Figure 4, where the production of the 50K fragment is plotted vs. time of tryptic digestion of cardiac acto–S-1 in the presence and absence of MgADP. Comparable proteolysis of skeletal acto–S-1 is not affected by the addition of MgADP (Muhlrad & Hozumi, 1982).

Binding experiments employing a Beckman airfuge reveal that the effect of MgADP on the 50K/20K junction in cardiac acto–S-1 cannot be attributed to partial dissociation of this protein complex. Comparisons of the dissociation of skeletal and cardiac acto–S-1 complexes by 2 mM MgADP show that under the conditions of our trypsin digestion experiments, the cardiac acto–S-1 complex is dissociated to the same or a smaller extent than the skeletal complex (approximately 10%). This is in agreement with the binding constants of cardiac and skeletal S-1 to actin in the presence of MgADP (Siemankowski & White, 1984; Greene & Eisenberg, 1980).

As judged by their actin-activated ATPase activity (see Materials and Methods), our cardiac S-1 preparations resemble similar preparations in the literature. Moreover, in support of our observations on the proteolysis of acto–S-1, it should also be mentioned that tryptic cleavage of rigor complexes of bovine cardiac myofibrils showed significant cleavage of the 50K/20K junction. Addition of 2 mM MgADP to such a system (plus appropriate inhibitors, i.e., AP_5A, glucose, and hexokinase) resulted in the increased cleavage of this junction (unpublished results). Thus, we conclude that the observed cleavage of the 50K/20K junction in the cardiac acto–S-1 complex and the acceleration of the rate of its cleavage by MgADP reflect intrinsic properties of the cardiac myosin head.

Discussion

In this study, we have used the method of limited tryptic digestion to compare and contrast the substructure of bovine cardiac myosin S-1 with that of skeletal myosin S-1. The tryptic cleavage of rabbit skeletal myosin S-1 yields three fragments, the 25K, 50K, and 20K peptides. This cleavage is altered both by the binding of actin, which protects the 50K/20K junction (Yamamoto & Sekine, 1979; Mornet et al., 1979), and by the binding of nucleotides (Mulhrad & Hozumi, 1982). As has been reported by others, trypsin cleaves cardiac myosin S-1 to yield the same 25K, 50K, and 20K peptides (Flink & Morkin, 1982; Klotz et al., 1983; Korner et al., 1983). This indicates that cardiac S-1, in analogy to its skeletal counterpart, is organized into three protease-resistant fragments connected by open linker peptides (Karn et al., 1983; Applegate & Reisler, 1983; Mornet et al., 1984) and agrees well with the anticipated overall structural similarity between the cardiac and skeletal myosin heads.

Particularly intriguing are the similar rates of proteolysis at the 50K/20K junctions in cardiac and skeletal myosin S-1. Sequence divergence in the linker peptide region spanning residues 630 through 652 (Karn et al., 1983) results in more than 70% amino acid mismatches between the rabbit cardiac and skeletal proteins (Kavinsky et al., 1984; Capony & Elzinga, 1981) or between nematode and rabbit skeletal myosins (Karn et al., 1983). The remaining portions of the 20K fragments in cardiac and skeletal S-1 show greater than 80% sequence homology. The fact that such major substitutions may occur in the 50K/20K junction, and yet have no apparent impact on the rate of its proteolytic cleavage, suggests a random coil structure of the linker peptide. It also may be argued that sequence divergency in the linker peptide does not necessarily point to its functional insignificance. In fact, its random coil character could be instrumental in generating a flexible link between different domains of the myosin head (Karn et al., 1983; Applegate & Reisler, 1983; Mornet et al., 1984; Muhlrad & Morales, 1984).

Differences between the cardiac and skeletal myosin S-1 are also apparent. First, the digestion of bovine cardiac S-1 proceeds at a 2-fold faster rate than that of the rabbit skeletal protein. We document here that this difference is due entirely to a much faster rate of tryptic attack at the 25K/50K junction. Extrapolating from the general protease susceptibility of this linker peptide (Applegate & Reisler, 1983, 1984; Mornet et al., 1984), and the sequence divergence in this region between skeletal and nematode myosins, we may anticipate substantial substitutions also between the cardiac and skeletal proteins. In contrast to the 50K/20K junctions, the expected substitutions in the 25K/50K linker peptide have a large effect on the rate of its proteolysis. Thus, in spite of the "open" character of the 25K/50K peptide linker, sequence substitutions appear to change at least some of its local or overall properties.

The second important difference we note is in the effect of actin on the tryptic cleavage of cardiac and skeletal S-1. While the cleavage of the 50K/20K junction in bovine cardiac S-1 is indeed inhibited an order of magnitude by the binding of actin, the inhibition is not nearly as complete as that seen in skeletal S-1. Furthermore, addition of 2 mM MgADP renders this junction in cardiac acto–S-1 more accessible to trypsin. We have tested and discarded the possibility that the tryptic cleavage of the 50K/20K junction in cardiac acto–S-1 is artifactual. First, binding experiments carried out in a Beckman airfuge demonstrate that our cardiac S-1 preparations bind actin at least as well as the skeletal S-1 preparations, both in the presence and in the absence of MgADP. This discounts the possibility that the observed differences in the production of the 20K and the 50K cardiac and skeletal peptides arise from the tryptic cleavage of free, "unbound", cardiac S-1. Second, this junction is cleaved in rigor complexes of cardiac myofibrils as well, and moreover, its cleavage in myofibrils is also accelerated by the addition of MgADP. We thus conclude that our results indicate intrinsic differences in the

substructure of the cardiac acto–S-1 compared to that of the skeletal complex.

At present, it is premature to speculate whether this difference is limited to the linker peptide regions and their reciprocal communication with actin and actin binding sites on S-1 or involves the acto–S-1 interface as well. Although the distinction between these two possibilities might be somewhat artificial, further characterization of the cardiac acto–S-1 complex is clearly needed. The fact that kinetic parameters of the interaction between actin, ADP, and S-1 are significantly different for the cardiac and skeletal myosins (Siemankowski & White, 1984) points to functional implications of their divergent substructures.

Registry No. MgADP, 7384-99-8.

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