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Proteolysis of Smooth Muscle Myosin by *Staphylococcus aureus* Protease: Preparation of Heavy Meromyosin and Subfragment 1 with Intact 20 000-Dalton Light Chains†

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ABSTRACT: The proteolysis of gizzard myosin by *Staphylococcus aureus* protease produces both heavy meromyosin and subfragment 1 in which the 20 000-dalton light chains are intact, and conditions are suggested for the preparation of each. Cleavage of the myosin heavy chain to produce subfragment 1 is dependent on the myosin conformation. Proteolysis of myosin in the 10S conformation yields predominantly heavy meromyosin, and myosin in the 6S conformation yields mostly subfragment 1 and some heavy meromyosin. Two sites are influenced by myosin conformation, and these are located at approximately 68 000 and 94 000 daltons from the N-terminus of the myosin heavy chain. The latter site is thought to be located at the subfragment 1–subfragment 2 junction, and cleavage at this site results in the production of subfragment 1. The time courses of phosphorylation of both heavy meromyosin and subfragment 1 can be fit by a single exponential. The actin-activated Mg^{2+}-ATPase activity of heavy meromyosin is markedly activated by phosphorylation of the 20 000-dalton light chains. From the actin dependence of Mg^{2+}-ATPase activity the following values are obtained: for phosphorylated heavy meromyosin, $V_{max} \sim 5.6\ s^{-1}$ and K_a (the apparent dissociation constant for actin) ~ 2 mg/mL; for dephosphorylated heavy meromyosin, $V_{max} \sim 0.2\ s^{-1}$ and $K_a \sim 7$ mg/mL. The actin-activated ATPase activity of subfragment 1 is not influenced by phosphorylation, and V_{max} and K_a for both the phosphorylated and dephosphorylated forms are $0.4\ s^{-1}$ and 5 mg/mL, respectively. The Mg^{2+}- and Ca^{2+}-ATPase activities of subfragment 1 are distinct from those of heavy meromyosin, and the Mg^{2+}-ATPase activity of subfragment 1 is not affected by phosphorylation. It is clear from these results that the phosphorylation of the 20 000-dalton light chain of subfragment 1 is not required for actin-activated ATPase activity. It is suggested that regulation via phosphorylation involves the interaction of the myosin heads with other parts of the molecule, possibly in the subfragment 2 region.

It is accepted that phosphorylation–dephosphorylation of the two 20 000-dalton light chains of myosin is involved in the regulation of contractile activity in smooth muscle (Adelstein & Eisenberg, 1980; Walsh & Hartshorne, 1982). Initiation of contraction and an increase in the actin-activated ATPase activity of myosin require phosphorylation, and the reverse process, i.e., dephosphorylation, accompanies relaxation and the loss of actin-activated ATPase activity (Güth & Junge, 1982). Although this general pattern for the function of myosin phosphorylation is well documented, its precise in vivo role is still unresolved. There are suggestions that in order to explain the observed biochemical and physiological data, additional Ca^{2+}-dependent regulatory mechanisms must be implicated (Marston, 1982; Murphy & Mras, 1983). Clearly the effects of light chain phosphorylation must be understood at the molecular level before one can evaluate its physiological role.

Recently it has been shown that monomeric smooth muscle myosin can exist in two distinct conformations (Suzuki et al., 1982) referred to as 10S and 6S to reflect their differences in sedimentation rates. The large conformational change is due to an apparent folding of the molecule as visualized by electron microscopy (Onishi & Wakabayashi, 1982; Trybus et al., 1982; Craig et al., 1983). Several factors influence the 10S–6S transition, including the ionic strength of the solvent and the $MgCl_2$ concentration (Ikebe et al., 1983), but of particular interest is the observation that phosphorylation of myosin favors the extended 6S form (Trybus et al., 1982; Craig et al., 1983; Onishi et al., 1983). Associated with the conformational change of myosin there is an alteration of ATPase activities, and it was shown that the 10S and 6S states are characterized by distinct enzymatic properties (Ikebe et al., 1983). This relationship prompted the suggestion that the

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6S–10S transition, or some component of it, could be involved in the regulatory mechanism of smooth muscle (Ikebe et al., 1983).

Two concerns raised in connection with this hypothesis were (1) that measurements of the 6S–10S transition were restricted to monomeric myosin and it is not known whether an equivalent transition can occur in filamentous myosin such as found in the physiological state (Somlyo et al., 1981), and (2) that heavy meromyosin (HMM)[1] is regulated by light chain phosphorylation (Sellers et al., 1981; Ikebe et al., 1981, 1982) but cannot form the looped 10S conformation since the tail portion of the molecule is missing. A technique that proved useful in providing some information on these points is that of limited proteolysis. Onishi & Watanabe (1984) found that the 10S conformation of gizzard myosin is more resistant to papain hydrolysis than the 6S form. Using both papain and *Staphylococcus aureus* protease digestion, we showed that the dephosphorylated form of filamentous myosin and that of HMM were more resistant to proteolysis than the phosphorylated forms (Ikebe & Hartshorne, 1984). The region of the myosin molecule that appears to be influenced by conformation is the head–neck junction rather than the HMM–LMM junction. This suggested that the 10S–6S transition induced changes in the S1–S2 part of the molecule and that these could be involved in determining the enzymatic properties of myosin. One method of investigating this possibility is to compare the properties of S1 to those of HMM and myosin. However, smooth muscle S1, made by either papain or trypsin digestion, does not contain a phosphorylation site (Jakes et al., 1976; Sobieszek & Small, 1976; Okamoto & Sekine, 1978; Seidel, 1978, 1980), and it is not possible to decide whether the Ca^{2+}-insensitive or unregulated acto-S1 ATPase activity (Sobieszek & Small, 1976; Seidel, 1980) is due to the lack of the phosphorylation site or the removal of the S1–S2 region. During the study on the proteolysis of 10S and 6S myosins (Ikebe & Hartshorne, 1984) it was found that *S. aureus* protease produces S1 and HMM, both which contain intact 20 000-dalton light chains. Therefore, the effect of phosphorylation on the actin-activated ATPase activity of S1 can now be evaluated.

In addition since the *S. aureus* protease S1 is a more valid representation of the myosin head, it could be of use in reconstruction and topography studies. The resistance of the 20 000-dalton light chains to proteolysis by *S. aureus* protease compared to α-chymotrypsin also facilitates the preparation of HMM with both phosphorylation sites intact.

This paper describes the preparation of S1 and HMM following hydrolysis of gizzard myosin by *S. aureus* protease and also presents a partial characterization of the physical and enzymatic properties of the two products.

Materials and Methods

Proteins were prepared from various sources by the following procedures: myosin from frozen turkey gizzards (Persechini & Hartshorne, 1983); myosin light chain kinase from frozen turkey gizzards (Walsh et al., 1983); calmodulin from frozen bull and goat testes (Walsh et al., 1983); actin from rabbit skeletal muscle (Driska & Hartshorne, 1975); HMM from gizzard myosin by proteolysis with α-chymotrypsin (Onishi & Watanabe, 1979); S1 from gizzard myosin by proteolysis with papain, 0.2 mg/mL (Margossian & Lowey, 1973).

Papain (type III) and α-chymotrypsin, 1-chloro-3-(tosylamido)-7-amino-2-heptanone treated (type VII), were obtained from Sigma Chemical Co., and *S. aureus* protease was obtained from Pierce Chemical Co. The papain was activated before use by incubating for 1 h at 35 °C in 50 mM Tris-HCl (pH 7.5), 0.5 mM EDTA, and 20 mM dithiothreitol. The following procedures were used to stop proteolysis: for *S. aureus* protease and α-chymotrypsin the addition of diisopropyl fluorophosphate (stock solution 0.1 M in 2-propanol) to 1 mM; for papain the addition of iodoacetic acid to 5 mM. Proteolysis with *S. aureus* protease was carried out at 25 °C under the conditions given in the figure legends. It is assumed that under these conditions cleavage of certain glutamyl bonds occurs. In the presence of 50 mM potassium phosphate (pH 7.6) both glutamyl and aspartyl bonds are cleaved (Drapeau, 1977), and the proteolysis products from myosin are considerably more numerous and complex. Thus, proteolysis in the presence of phosphate buffer should be avoided.

To establish the effects of different solvent conditions on the patterns of proteolysis of myosin by *S. aureus* protease, a Perkin-Elmer Series 4 high-performance liquid chromatography system was used (see Figure 1). The digests (0.25 mL) were applied to a TSK G4000 SW column (60 × 0.75 cm) in series with a TSK G2000 SW column (30 × 0.75 cm). The latter was necessary to separate ATP from the S1 peak. The following criteria were used to identify the components in each peak: The elution positions of HMM and S1 were approximated with α-chymotryptic HMM and papain S1; $NaDodSO_4$–polyacrylamide gel electrophoresis was carried out on each fraction, which allowed the identification of proteins associated with unique peptides [e.g., the heavy chains of intact myosin, the 130- and 126-kDa peptides for 10S–HMM, the 26-kDa peptide of S1 (both for *S. aureus* protease and papain S1 preparations)]; solubility at low ionic strength was ascertained (e.g., the proteins of peak 1 from HPLC were insoluble at low ionic strength, and those of peaks 2 and 3 were soluble); the ATPase characteristics for each peak were established, and these are as described in the text.

On a more preparative scale HMM and S1 were isolated by chromatography on Sephacryl S-300 (see Figure 2). For the experiments described in this paper HMM was prepared from dephosphorylated 10S myosin (~ 40 mg) by proteolysis at 25 °C for 45 min with *S. aureus* protease (1:100 protease–myosin, w/w) in 0.2 M KCl, 1 mM $MgCl_2$, 30 mM Tris-HCl (pH 7.5), 1 mM dithiothreitol, 0.2 mM EGTA, and 2 mM ATP. Diisopropyl fluorophosphate was added to 1 mM and the $MgCl_2$ concentration adjusted to 15 mM. Following a brief dialysis (approximately 1 h) at 0 °C vs. 30 mM Tris-HCl (pH 7.5), 15 mM $MgCl_2$, and 0.2 mM dithiothreitol, the insoluble protein was removed by centrifugation at 180000g_{av} for 45 min. The supernatant was adjusted to 0.3 M KCl and applied (5 mL) to a Sephacryl S-300 column (2.4 × 60 cm) equilibrated with 0.3 M KCl, 30 mM Tris-HCl (pH 7.5), and 0.2 mM dithiothreitol. Fractions of 3 mL were usually collected at a flow rate of approximately 20 mL/h. The fractions that were pooled were based on the $NaDodSO_4$–polyacrylamide gel patterns (see Figure 2).

S1 was prepared from dephosphorylated 6S myosin by an identical procedure except that ATP was omitted from the solvent used for the digestion of 10S myosin by the *S. aureus* protease.

ATPase activities were measured at 25 °C under the conditions given in the figure legends. The ATPase reactions were

[1] Abbreviations: HMM, heavy meromyosin; S1, heavy meromyosin subfragment 1; S2, heavy meromyosin subfragment 2; LMM, light meromyosin; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; $NaDodSO_4$, sodium dodecyl sulfate; HPLC, high-performance liquid chromatography; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; kDa, kilodalton; ATPase, adenosinetriphosphatase.

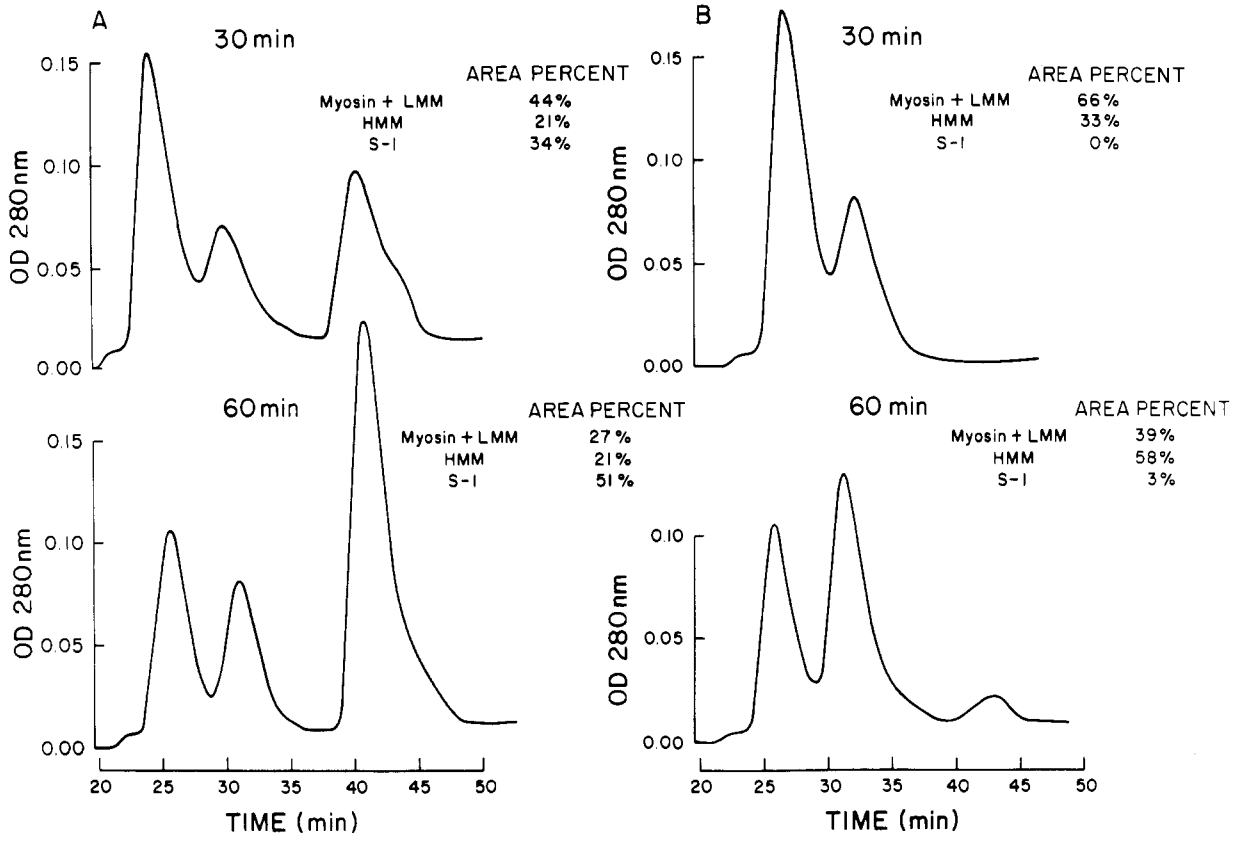

FIGURE 1: Elution profiles of high-performance liquid chromatography of myosin digested by *S. aureus* protease. Myosin (5 mg/mL) was phosphorylated to ~1.8 mol/mol of myosin in 0.2 M KCl and 1 mM $MgCl_2$ (as described under Materials and Methods). Dephosphorylated myosin was treated similarly, except that EGTA (2 mM) was included in the incubation medium. Phosphorylated (A) and dephosphorylated (B) myosins were digested at 25 °C with 1:100 protease–myosin (w/w ratio) for 30 and 60 min. For both myosin samples the proteolysis conditions were 0.2 M KCl, 2 mM ATP, 1 mM $MgCl_2$, and 30 mM Tris-HCl (pH 7.5). After proteolysis was stopped (see Materials and Methods) KCl was added to 0.4 M, and 0.25-mL aliquots were applied to the chromatography system.

stopped by the addition of an aliquot (0.2–0.5 mL) of the assay mixture to a stoppered plastic funnel (Isolab Quik-Sep column, code QS-P) containing 1 mL of 2% (w/w) activated charcoal (Sigma Chemical Co.) and 0.5 mL of 1 N perchloric acid and 0.35 M NaH_2PO_4. The samples were mixed, by a vortex mixer, kept on ice (to retard the acid-catalyzed hydrolysis of ATP), and filtered (facilitated by slight positive air pressure), and aliquots of the filtrate were subject to Čerenkov counting. Contamination of the [γ-^{32}P]ATP by inorganic phosphate ([^{32}P]P_i) was determined by this procedure at zero time or in the absence of myosin. For the calculation of enzymatic activities molecular weight values of 334 000 and 130 000 were used for HMM and S1, respectively.

Phosphorylation of the 20 000-dalton light chain was measured as described by Walsh et al. (1983). [γ-^{32}P]ATP was obtained from New England Nuclear. Myosin, HMM, and S1 were phosphorylated by incubation for 15 min at 25 °C with 10 μg/mL calmodulin, 10 μg/mL myosin light chain kinase, 1 mM [γ-^{32}P]ATP, 30 mM Tris-HCl (pH 7.5), and 0.1 mM $CaCl_2$. The KCl and $MgCl_2$ concentrations used were varied and given in the figure legends.

Electrophoresis was carried out on 7.5–20% polyacrylamide gradient slab gels in the presence of 0.1% $NaDodSO_4$ at 30 mA by using the discontinuous buffer system of Laemmli (1970). After staining with Coomassie Brilliant Blue R 250 (Sigma Chemical Co.) gels were scanned using a GS 300 scanning densitometer (Hoefer Scientific Instruments) attached to a LCI-100 Laboratory computing integrator (Perkin-Elmer). Subunit molecular weights were determined by using the following proteins and molecular weight values: myosin heavy chain (200 000), caldesmon (150 000), turkey gizzard myosin light chain kinase (130 000), bovine serum albumin (68 000), actin (42 000), smooth muscle myosin light chains (20 000 and 17 000), and components of rabbit skeletal muscle myofibrils.

Other procedures were as described by Walsh et al. (1982).

RESULTS

Proteolytic Cleavage of Myosin and the Preparation of HMM and S1. Phosphorylated and dephosphorylated gizzard myosins in 0.2 M KCl, 2 mM ATP, 1 mM $MgCl_2$, and 30 mM Tris-HCl (pH 7.5) were digested by *S. aureus* protease for 30 and 60 min. The digest was adjusted to 0.4 M KCl and was applied to exclusion chromatography on an HPLC system (see Materials and Methods). The results are shown in Figure 1. Under the conditions used for proteolysis dephosphorylated and phosphorylated gizzard myosins exist as 10S and 6S, respectively (Ikebe et al. 1983). The elution profile following hydrolysis of 6S myosin shows three peaks for both the 30- and 60-min samples (Figure 1A). The first peak contains mostly myosin and LMM plus an unidentified 160-kDa peptide, the second peak contains predominantly HMM, and the third peak contains S1. The percentage areas for each peak are given in Figure 1. In contrast the pattern of proteolysis for 10S myosin is quite different in that the extent of S1 liberation is markedly reduced (Figure 1B). After a 30-min proteolysis only the myosin and HMM peaks are observed, and after a 60-min digestion a third peak corresponding to S1 appears but comprises only a minor fraction of the total protein (Figure 1B). It can be estimated that the rate of S1 production is approximately 15 times slower for 10S compared to that of 6S myosin, and these results confirm our previous data

(Ikebe & Hartshorne, 1984). If it is assumed that S1 is derived from both HMM and intact myosin, then it may also be estimated that the rate of HMM production is similar for 6S and 10S myosins and suggests that the conformation of myosin, i.e., 6S or 10S, influences the sensitivity to proteolysis of a region of the molecule at the S1–S2 junction rather than at the HMM–LMM junction. Also it is shown that the preferred substrate of *S. aureus* protease for the preparation of HMM is 10S myosin and the preferred substrate for the preparation of S1, or S1 plus HMM, is 6S myosin.

The HPLC elution patterns following a 60-min digestion by *S. aureus* protease of dephosphorylated myosin (1:100 w/w ratio) in 0.2 M KCl and the absence of ATP and in 0.4 M KCl and 2 mM ATP (other conditions as given above) are similar to that shown in Figure 1A for 6S myosin. Under these conditions (i.e., 0.2 M KCl minus ATP and 0.4 M KCl plus ATP) dephosphorylated myosin forms the 6S conformation (Ikebe et al., 1983), and this reiterates the point made previously (Ikebe & Hartshorne, 1984) that the difference in sensitivity to proteolysis is not due directly to the phosphorylation level but rather is a reflection of the myosin conformation.

To isolate S1 and HMM in larger amounts, chromatography on Sephacryl S-300 was used. (The details of these procedures are given under Materials and Methods.) Chromatography profiles as examples of each preparation are shown in Figure 2. Under the solvent conditions used for the proteolysis and in the presence of ATP, dephosphorylated myosin exists in the 10S conformation, whereas in the same conditions and the absence of ATP the 6S conformation is favored (Ikebe et al., 1983). In both cases two peaks are resolved; the first contains HMM and the more retarded peak contains S1. However, the proportion of each peak is different for the two conformations. Proteolysis of 10S myosin by *S. aureus* protease produces mainly HMM, and proteolysis of 6S myosin yields predominantly S1 (Figure 2).

Peptide Composition of S1 and HMM. The composition of S1 obtained from both 6S and 10S myosins is similar and contains peptides of 68 and 26 kDa plus the two light chains (Figure 2). The HMM preparations isolated from 6S and 10S myosins, however, are different (Figure 2). HMM obtained from 10S myosin is composed of the following peptides: 130, 126, 68, 62, and 58 kDa plus the two light chains. The HMM from 6S myosin does not contain the two larger peptides (130 and 126 kDa). Despite the difference in peptide composition there is no detectable difference in enzymatic properties between the two preparations of HMM.

Between the HMM and S1 peaks peptides of 36 and 32 kDa were frequently detected, and these are thought to be derived from S2.

Phosphorylation of HMM and S1. From the gel electrophoresis patterns shown in Figure 2, it is evident that the 20 000-dalton light chain remains apparently intact in preparations of both HMM and S1. This is a particular advantage in the case of S1, since the method used most frequently in the past, i.e., papain hydrolysis of myosin, results in the loss of the phosphorylation sites. The resistance of the 20 000-dalton light chain to *S. aureus* protease digestion is also beneficial in the preparation of HMM. Conventionally the preparation of smooth muscle HMM is achieved by digestion of myosin with α-chymotrypsin. This procedure can yield a product in which the phosphorylation sites are intact (Seidel, 1978; Onishi & Watanabe, 1979; Sellers et al., 1981) although the 20 000-dalton light chains are subject to hydrolysis by α-chymotrypsin. A comparison of the effects of proteolysis

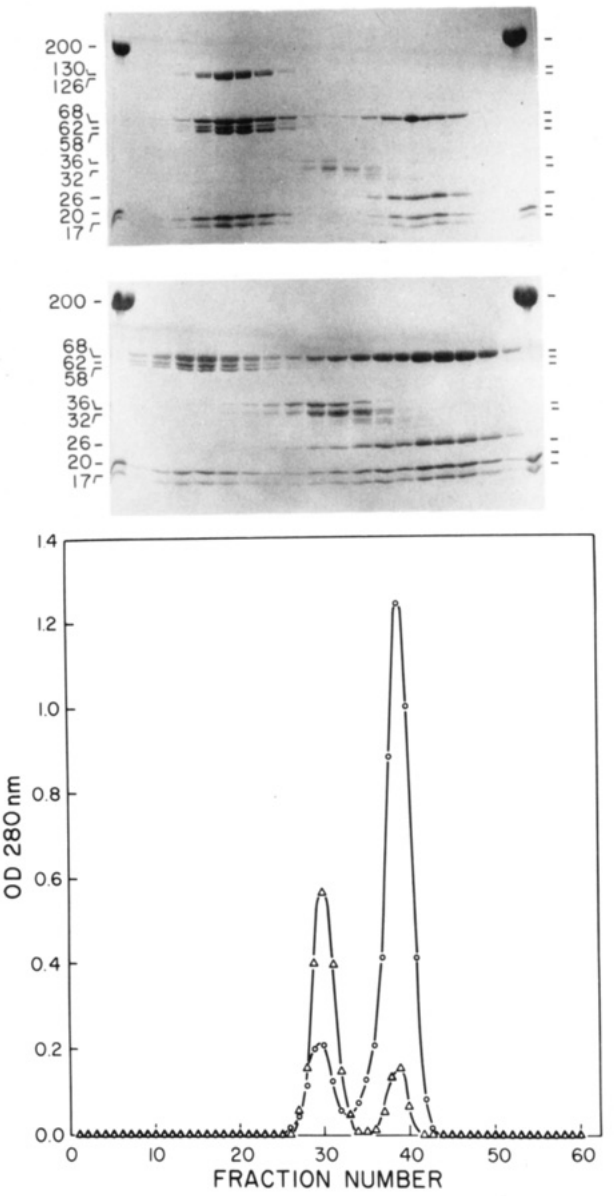

FIGURE 2: Elution profile of Sephacryl S-300 gel filtration of HMM and S1. Myosin (5 mL at 8 mg/mL) was treated as described under Materials and Methods in the presence of ATP (△) and in the absence of ATP (○), i.e., 10S and 6S myosin, respectively. The NaDodSO$_4$–polyacrylamide profiles are shown for 10S myosin (top) and 6S myosin (below). The two outside lanes on each gel are control myosin. For the top gel, lanes 3–19 are for successive fractions 26–42, respectively. For the bottom gel, lanes 2–19 are for successive fractions 26–43, respectively.

of myosin by *S. aureus* protease and by α-chymotrypsin is shown in Figure 3. The conditions of proteolysis were for α-chymotrypsin those used by Onishi & Watanabe (1979) and for *S. aureus* protease, 0.2 M KCl and the absence of ATP (see Figure 2). Under either condition, myosin exists in the 6S conformation. In both cases the weight ratio of protease to myosin was 1:100. The phosphorylation level measured after varying times of proteolysis by *S. aureus* protease, remains constant, whereas proteolysis by α-chymotrypsin results in a decrease in the number of phosphorylation sites (Figure 3A). This pattern is consistent with the estimated area of the 20 000-dalton light chain remaining after varying times of digestion for the two proteases (Figure 3B). Proteolysis by *S. aureus* protease does not result in an appreciable loss of light chain, in contrast to the marked reduction of 20 000-dalton light chain following hydrolysis by α-chymotrypsin. Both enzymes cause a relatively rapid cleavage of the myosin

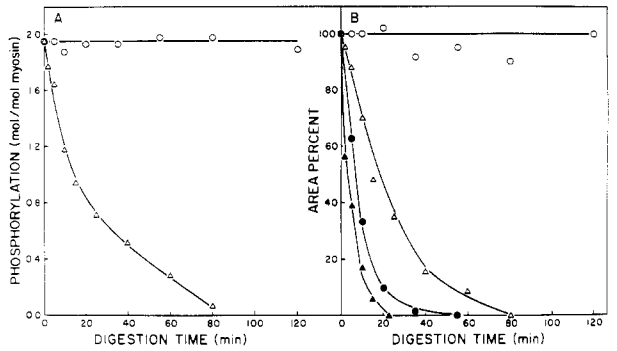

FIGURE 3: Proteolysis of gizzard myosin by *S. aureus* protease and by α-chymotrypsin. Myosin, 5 mg/mL, in 0.2 M KCl, 1 mM $MgCl_2$, 30 mM Tris-HCl (pH 7.5), and 1 mM dithiotheitol was digested at 25 °C with *S. aureus* protease (1:100 protease–myosin, w/w ratio) for varying times. Myosin, 5 mg/mL, in 0.6 M KCl, 50 mM potassium phosphate (pH 7.0), 1 mM EDTA, and 1 mM dithiothreitol was also digested with α-chymotrypsin (1:100 w/w ratio). Conditions for determining the maximum extent of phosphorylation were 1 mg/mL myosin, 10 mM $MgCl_2$, 30 mM Tris-HCl (pH 7.5), 0.1 mM $CaCl_2$, 10 μg/mL calmodulin, and 10 μg/mL myosin light chain kinase, for 15 min at 25 °C. The level of remaining phosphorylation sites is shown in (A) following digestion with α-chymotrypsin (Δ) and with *S. aureus* protease (O). $NaDodSO_4$–polyacrylamide gels from each time point in (A) were scanned, and the percentage area of the myosin heavy chain (●, ▲) and that of the 20 000-dalton light chain (O, Δ) were determined. These are shown in (B): for *S. aureus* protease (O, ●) and for α-chymotrypsin (Δ, ▲).

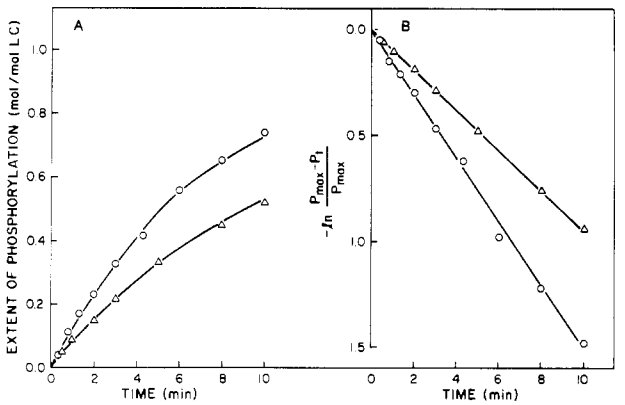

FIGURE 4: Time course of phosphorylation of S1 and HMM. Conditions: S1, or HMM, 0.2 mg/mL, in 100 mM KCl, 1 mM $MgCl_2$, 1 mM [γ-^{32}P]ATP, 30 mM Tris-HCl (pH 7.5), 10 μg/mL calmodulin, and 0.15 μg/mL myosin light chain kinase at 25 °C. The maximum extent of phosphorylation (P_{max}) was determined after 15 min under similar conditions with 10 μg/mL myosin light chain kinase. (A) Time course of phosphorylation for S1(O) and HMM (Δ). (B) Semilogarithmic plot of phosphorylation data for S1 (O) and HMM (Δ).

heavy chain (Figure 3B). Similar data were obtained when the hydrolysis by α-chymotrypsin was carried out under identical conditions with those used for the *S. aureus* protease digestion.

The site of phosphorylation by myosin light chain kinase was restricted to the 20 000-dalton light chain for both HMM and S1, (prepared by the *S. aureus* protease procedures) as determined by autoradiography of the $NaDodSO_4$–polyacrylamide gels (data not shown).

The time courses of phosphorylation for HMM and S1 are shown in Figure 4A. The data for both HMM and S1 are fitted by a single exponential (Figure 4B). The rate of phosphorylation for S1 was slightly faster (~1.5 times) than that for HMM.

Enzymatic Activities of HMM and S1. The actin dependence of Mg^{2+}-ATPase activity for dephosphorylated and

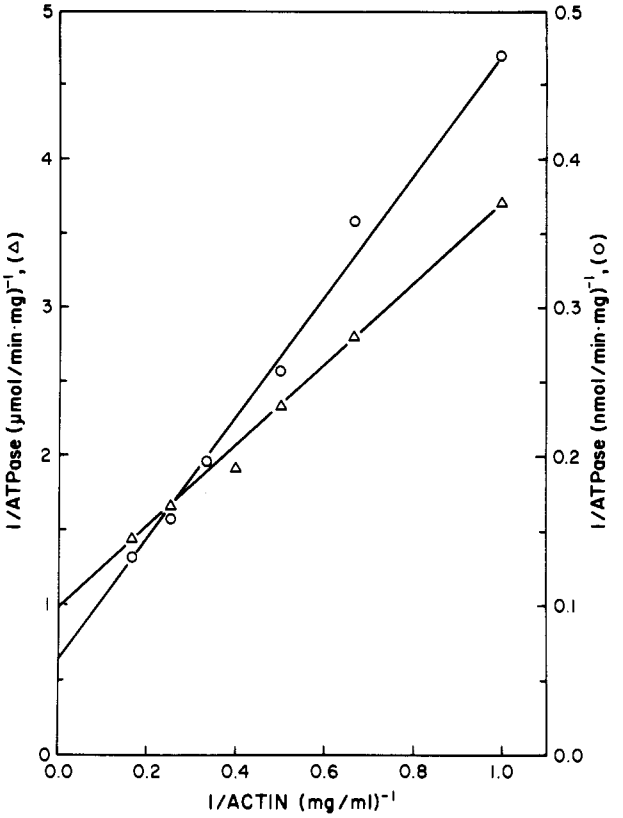

FIGURE 5: Double-reciprocal plot for the actin dependence of Mg^{2+}-ATPase activity of HMM. Conditions: 0.1 mg/mL HMM, 20 mM KCl, 1 mM $MgCl_2$, 30 mM Tris-HCl (pH 7.5), either 0.1 mM $CaCl_2$ (Δ) or 1 mM EGTA (O), and varied skeletal muscle F-actin. Phosphorylated HMM (Δ); dephosphorylated HMM (O). Note difference in units for ordinates. The Mg^{2+}-ATPase activity of HMM alone for the phosphorylated [~2 nmol/(min·mg)] and dephosphorylated [~0.6 nmol/(min·mg)] species was subtracted from each point.

phosphorylated (1.8 mol of phosphate/mol of HMM) HMM is shown in Figure 5. As anticipated the acto-HMM ATPase activity is markedly increased by phosphorylation; the V_{max} values for acto dephosphorylated HMM and for acto phosphorylated HMM are 33 (~0.18 s^{-1}) and 1000 nmol/(min·mg) (~5.57 s^{-1}), respectively. The extrapolated values for K_a are 2 mg/mL actin for phosphorylated HMM and 7 mg/mL actin for dephosphorylated HMM.

The actin dependence of Mg^{2+}-ATPase activity for dephosphorylated and phosphorylated (~0.9 mol of phosphate/mol of S1) S1 is shown in Figure 6. In contrast to the situation with acto-HMM the actin-activated ATPase activity of S1 is not influenced by light chain phosphorylation. Values for V_{max} and K_a were estimated to be 200 nmol/(min·mg) (0.44 s^{-1}) and 5 mg/mL actin, respectively.

It has been shown that the ATPase activities of gizzard myosin are sensitive to changes in ionic strength (Onishi et al., 1978) and that the decrease in activity for the Ca^{2+}- and Mg^{2+}-ATPase of myosin, which occurs as the ionic strength is reduced, is correlated to a shift in myosin conformation from 6S to 10S (Ikebe et al., 1983). To determine whether these properties of intact myosin are retained in our preparations of HMM and S1, the ionic strength dependence of the Mg^{2+}- and Ca^{2+}-ATPase activities was measured.

The KCl dependence of the Mg^{2+}-ATPase activities for phosphorylated (~0.9 mol of phosphate/mol of S1) and dephosphorylated S1 is shown in Figure 7. As the ionic strength is reduced, the activities are increased, and there is no influence of phosphorylation. This behavior is different from that shown

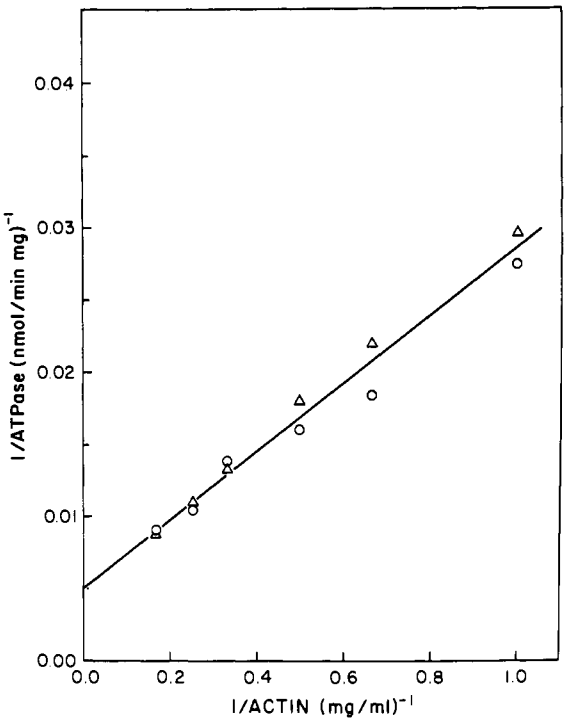

FIGURE 6: Double-reciprocal plot for the actin dependence of Mg^{2+}-ATPase activity of S1. Conditions as in Figure 5. Phosphorylated S1 (△); dephosphorylated S1 (○). The Mg^{2+}-ATPase activity of S1 alone [~9 nmol/(min·mg) for both phosphorylated and dephosphorylated forms] was subtracted from each point.

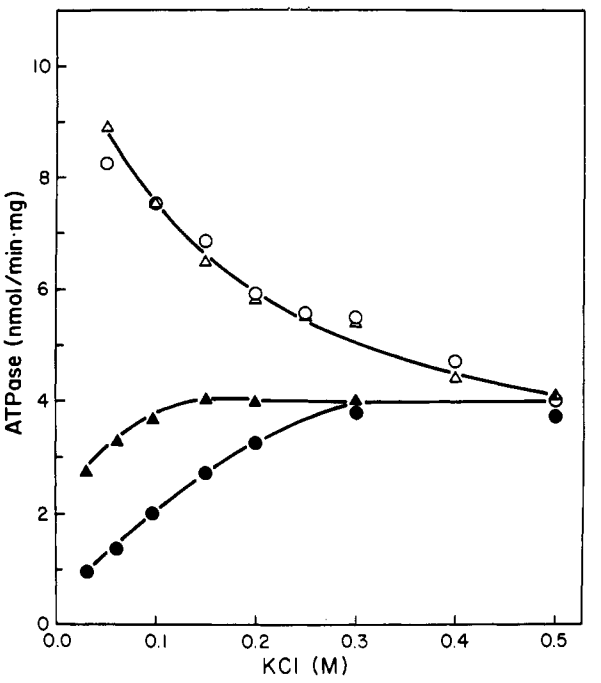

FIGURE 7: KCl dependence of Mg^{2+}-ATPase activity for S1 and for HMM. Conditions: 0.1 mg/mL S1 or HMM, 10 mM $MgCl_2$, 0.3 mM [γ-^{32}P]ATP, 30 mM Tris-HCl (pH 7.5), and either 0.1 mM $CaCl_2$ (△, ▲) or 1 mM EGTA (○, ●). Phosphorylated S1 (△), phosphorylated HMM (▲), dephosphorylated S1 (○), and dephosphorylated HMM (●).

by HMM (Figure 7). The Mg^{2+}-ATPase activities for phosphorylated (1.8 mol of P/mol of HMM) and dephosphorylated HMM both decrease at lower ionic strengths. The effect of phosphorylation is to shift the activity depression to lower KCl concentrations. Thus, at a fixed KCl concentration phosphorylation of HMM would increase the Mg^{2+}-ATPase activity, and this would be more noticeable at lower ionic strengths (below 0.3 M KCl). Similar effects of phosphorylation are found for the Mg^{2+}-ATPase activity of intact myosin (Ikebe et al., 1983).

The influence of KCl on the Ca^{2+}-ATPase activities of S1 and HMM also is different (data not shown). Below 0.3 M KCl the Ca^{2+}-ATPase activity of HMM is decreased and, in this respect, resembles myosin. In contrast the Ca^{2+}-ATPase activity of S1 is increased as the ionic strength is reduced.

Discussion

The major advantage associated with the use of *S. aureus* protease is the resistance to hydrolysis of the 20 000-dalton light chains. This is particularly useful in the case of S1 since it allows a preparation in which the phosphorylation site is intact. [In a preliminary report (Nath et al., 1982) it was noted that papain S1 could be prepared with an intact 20 000-dalton light chain; however, the details of this procedure have not been presented.] The significance of this is that the effects of phosphorylation on the ATPase activities of S1 can be evaluated. It is important to establish whether each head can be regulated independently or if other interactions, e.g., head–head or S1–S2, are involved. It is clear from the results presented in this paper that phosphorylation of the 20 000-dalton light chain of S1 does not influence the measured ATPase activities. Most important is the finding that phosphorylation is not required for the activation by actin of the Mg^{2+}-ATPase activity of S1. This would suggest that the individual heads do not act as independent units and that other parts of the myosin molecule are involved in the regulatory mechanism. It will be interesting to compare the properties of gizzard myosin and its fragments to those of scallop myosin, since it was found with the latter that S1 is not regulated by Ca^{2+} but single-headed myosin is Ca^{2+} dependent, suggesting a role for S2 in the regulatory mechanism of scallop myosin (Stafford et al., 1979).

In the preparation of HMM the benefit of using the *S. aureus* protease procedure is less obvious since regulated HMM can be prepared by digestion with α-chymotrypsin (Seidel, 1978, 1980; Onishi & Watanabe, 1979; Sellers et al., 1981). However, the 20 000-dalton light chains are degraded by α-chymotrypsin (Seidel, 1978; Onishi & Watanabe, 1979; see Figure 3), and to retain a full complement of phosphorylation sites in α-chymotryptic HMM requires a judicious choice of experimental conditions. For the preparation of HMM by *S. aureus* protease the time of proteolysis is less critical, and for this reason the preparation is simpler. The yield of actin-activated HMM from the *S. aureus* protease procedure also is generally higher than for the α-chymotrypsin preparation; for example, the amount of HMM obtained from 10S myosin in the example given in Figure 2 was about 30% of the parent myosin.

In general, the *S. aureus* protease HMM is similar in its enzymatic properties to the HMM produced by hydrolysis with α-chymotrypsin. Our values for the actin-phosphorylated HMM interaction, V_{max} and K_a (the apparent dissociation constant for actin determined from ATPase measurements), are 5.7 s^{-1} at 25 °C and 2 mg/mL, respectively. Values obtained with phosphorylated α-chymotryptic HMM are V_{max} ~ 5 s^{-1} at 28 °C and K_a ~ 5.5 mg/mL (Onishi & Watanabe, 1979), V_{max} ~ 2.5 s^{-1} at 20 °C and K_a ~ 3.2 mg/mL (Ikebe et al., 1981), and V_{max} ~ 1.89 s^{-1} at 25 °C and K_a ~ 1.6 mg/mL (Sellers et al., 1982). Some variation in the values for K_a is expected since these parameters were derived at different ionic strengths, and it is usually assumed that increasing ionic strength reduces the affinity of actin for myosin.

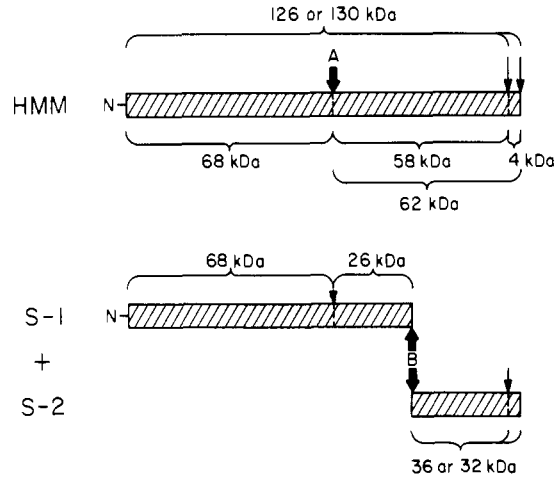

FIGURE 8: Schematic showing sites of hydrolysis by *S. aureus* protease. The peptide compositions of HMM, S1, and S2 are given. Sites A and B are those thought to be influenced by the conformation of myosin. Cleavage at site B is required for the release of S1. For other details see text. Similar major heavy chain peptides, i.e., approximately 68 and 26 kDa, were found for *S. aureus* protease S1 and for papain S1.

With respect to other properties the α-chymotryptic HMM and the *S. aureus* protease HMM also are similar. The influence of ionic strength on the Ca^{2+}- and Mg^{2+}-ATPase activities is comparable, and both preparations show an effect of phosphorylation on these enzymatic parameters that is thought to reflect a conformational transition. The latter can also be detected by limited proteolysis (Ikebe & Hartshorne, 1984), and HMM prepared by either protease is more resistant to degradation in the dephosphorylated form.

The values obtained for the actin dependence of the Mg^{2+}-ATPase activity of phosphorylated and dephosphorylated S1 are $V_{max} \sim 0.44$ s^{-1} and $K_a \sim 5$ mg/mL. Under similar conditions (temperature and ionic strength) values obtained by others using papain S1 are $V_{max} \sim 0.7$ s^{-1} and $K_a \sim 2.5$ mg/mL (Marston & Taylor, 1980) and $V_{max} \sim 0.75$ s^{-1} and $K_a \sim 3$ mg/mL (Greene et al., 1983). It is not known whether the slightly lower value for V_{max} obtained with our procedure is due to the presence of the 20000-dalton light chain or is within the range expected for experimental variation.

A tentative assignment of peptides identified in HMM and S1 prepared by *S. aureus* protease hydrolysis of gizzard myosin is shown in Figure 8. (The 20000- and 17000-dalton light chains are not shown, but it is assumed that they are present in both the HMM and S1 preparations.) The heavy chain of HMM is about 130 kDa. Often this is clipped, by about 4 kDa, from the C-terminal end. A cleavage site is also located in about the center of the HMM heavy chain (site A of Figure 8). This site is interesting in that it appears to be more accessible, or labile, in the 6S state, and the parent peptides, i.e., 130 and 126 kDa, are found only in HMM prepared from 10S myosin. However, cleavage at site A does not markedly influence the properties of HMM, and HMM from 10S myosin (containing 130-, 126-, 68-, 62-, and 58-kDa peptides) and that from 6S myosin (containing 68-, 62-, and 58-kDa peptides) are both dependent on phosphorylation for activation of actin-activated ATPase activity. The critical site, or region of the molecule, that is subject to control by phosphorylation, therefore, is retained in both HMM preparations.

Generation of the S1 fragment involves proteolysis at a distinct site, designated B in Figure 8. This is the most interesting of the *S. aureus* protease hydrolysis sites since its cleavage is markedly dependent on conformation, and the release of S1 from 10S myosin is considerably reduced compared to 6S myosin. The location of site B is thought to be at the S1–S2 junction, and therefore, this region of the molecule is influenced by the 10S–6S transition. In addition, the hydrolysis of site B results in the conversion from a phosphorylation-dependent molecule, HMM, to an unregulated moiety, S1. On the basis of the preliminary results of Nath et al. (1982) and the situation observed with scallop myosin (Stafford et al., 1979), it is likely that the S1–S2 junction of gizzard myosin is implicated in the regulatory mechanism. Marianne-Pepin et al. (1983) analyzed the tryptic peptides of myosin and reached a similar conclusion. They suggested that a 66-kDa tryptic peptide, equivalent to our 62- or 58-kDa peptides, contained the S1–S2 junction and that this was cleaved by papain in the release of S1 to give fragments of 26 and 37 kDa. Under certain conditions it was proposed that the 66-kDa peptide is inhibitory to actin-activated ATPase activity and could therefore be involved in the phosphorylation-dependent regulatory mechanism (Marianne-Pepin et al., 1983).

In general the patterns of peptide release from gizzard myosin by other proteases are compatible with the results presented here. It was reported that the heavy chain of α-chymotryptic HMM, approximately 130 kDa, was cleaved in half on further digestion (Seidel, 1980; Okamoto & Sekine, 1981). For the proteolysis of gizzard myosin by papain, Nath et al. (1982) identified two major sites of cleavage, at 67 and 90 kDa from the N-terminus, and Onishi & Watanabe (1984) found that two sites, 72 and 94 kDa from the N-terminus, were masked in the 10S conformation. Presumably the two sites identified in these reports are equivalent and would correspond to sites A and B (Figure 8) found with *S. aureus* protease.

Registry No. ATPase, 9000-83-3; *Staphylococcus aureus* protease, 82062-91-7.

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Control of Energy Production in the Heart: A New Function for Fatty Acid Binding Protein

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ABSTRACT: The quantitative subcellular distribution of the fatty acid binding protein (FABP) in heart muscle is reported for the first time. A gradient-like distribution according to the following pattern was observed: 6.96 mg·mL^{-1} on the myofibrils, 2.77 mg·mL^{-1} in the spaces surrounding the mitochondria, and 2.21 mg·mL^{-1} in the mitochondria. This heterogeneous distribution suggests that the local in vivo concentration of FABP might fluctuate as a function of time. The consequences of these possible fluctuations, particularly in the mitochondrial vicinity, were analyzed in an in vitro system containing a fixed concentration of cardiac mitochondria and stearic acid but variable concentrations of FABP. Competition for the fatty acid was observed between the mitochondrial membranes and the binding sites on the protein. Maximal binding of fatty acid to FABP was detected in the range of FABP concentration between 1 and 3 mg·mL^{-1}. Remarkably, in this concentration range, two emerging peaks of β-oxidative activity were also detected. As a major conclusion, it appears that the fatty acid pool, bound to FABP, is the source of fatty acid providing the β-oxidative system with substrate. The mechanism of fatty acid transfer from this pool toward the β-oxidative system remains an open question. However, it is suggested that a gradient-like distribution of FABP in the mitochondrial vicinity leads to the coexistence of multispecies of the protein by self-aggregation. Only two of these species seem to be involved in this fatty acid transfer. As a consequence, a strong modulation of fatty acid β-oxidation rate is observed in isolated mitochondria when the concentrations of these two species are allowed to fluctuate. In conclusion, this unique cardiac fatty acid carrier, via its self-aggregation capacity and its in vivo gradient-like distribution, may act as a powerful effector in the regulation of heart energy.

A fatty acid binding protein (FABP)[1] characterized by a very high affinity for long-chain fatty acids was detected in the heart (Ockner et al., 1972). The total purification of the protein (Fournier et al., 1978) opened the door for detailed molecular studies of this novel and unique fatty acid carrier. A remarkable property of this protein soon became apparent; although the minimal molecular weight was determined to be about 12K, at least three other species coexisting at equilibrium by self-aggregation were detected by applying circular dichroism and electron spin resonance techniques (Fournier et al., 1983; Fournier & Rahim, 1983). The biological significance of the aggregation potential of the FABP species was scrutinized in a theoretical model analysis (Fournier et al., 1983). Strong modulations of membrane-bound fatty acid dependent enzyme activity were predicted for the case where one FABP species was selectively allowed to transfer fatty acids

[1] Abbreviations: FABP, fatty acid binding protein; ESR, electron spin resonance; 12-doxylstearate, 2-(10-carboxydecyl)-2-hexyl-4,4-dimethyl-3-oxyoxazolidine; EDTA, ethylenediaminetetraacetic acid disodium salt; IEF, isoelectric focusing; CoA, coenzyme A; TLC, thin-layer chromatography.

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