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INTERACTION OF RAT MUSCLE AMP DEAMINASE WITH MYOSIN

I. BIOCHEMICAL STUDY OF THE INTERACTION OF AMP DEAMINASE AND MYOSIN IN RAT MUSCLE

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Summary

AMP deaminase was completely solubilized from rat skeletal muscle with 50 mM Tris-HCl buffer (pH 7.0) containing KCl at a concentration of 0.3 M or more. The purified enzyme was found to be bound to rat muscle myosin or actomyosin, but not to F-actin at KCl concentrations of less than 0.3 M. Kinetic analysis indicated that 1 mol of AMP deaminase was bound to 3 mol of myosin and that the dissociation constant (K_d) of this binding was 0.06 μM. It was also shown that AMP deaminase from muscle interacted mainly with the light meromyosin portion of the myosin molecule. This finding differs from that of Ashby and coworkers on rabbit muscle AMP deaminase, probably due to a difference in the properties of rat and rabbit muscle AMP deaminase.

AMP deaminase isozymes from rat liver, kidney and cardiac muscle did not interact with rat muscle myosin. The physiological significance of this binding of AMP deaminase to myosin is discussed.

Introduction

AMP deaminase (AMP-aminohydrolase, EC 3.5.4.6), catalyzing the deamination of AMP to form IMP and ammonia, is widely distributed in animal tissues and is found in the highest concentration in skeletal muscle. It has been reported that ammonia production by muscle is proportional to the amount of work done by the muscle [1] and the reaction catalyzed by this enzyme provides the

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major source of ammonia in the muscle [2,3]. However, attempts to obtain direct evidence that this enzyme is involved in muscle contraction have been unsuccessful [4,5].

Lowenstein proposed that the purine nucleotide cycle, consisting of AMP deaminase, adenylosuccinate synthetase (IMP:L-aspartate ligase (GDP-forming), EC 6.3.4.4) and adenylosuccinase (adenylosuccinate AMP-lyase, EC 4.3.2.2), operated in the ammoniagenesis from amino acid in skeletal muscle [6] and he also suggested that this cycle was closely linked to glycolytic oscillation [7]. On the other hand, we reported previously that adenylosuccinate synthetase in muscle and the muscle type (M) enzyme in the liver were more sensitive to inhibition by fructose 1,6-diphosphate, a reaction product of phosphofructokinase (ATP:D-fructose-6-phosphate 1-phosphotransferase EC 2.7.1.11), than the liver type (L) enzyme in the liver, while the two former were less sensitive to inhibition by nucleotide mono- and diphosphates than the latter. On the basis of these findings, we proposed that the muscle enzyme and the type M enzyme might regulate glycolysis, while type L enzyme regulates de novo purine nucleotide synthesis [8—12]. We also found that muscle and type M adenylosuccinate synthetase interacted with F-actin [12]. It has been reported that glycolytic enzymes in the muscle, such as phosphofructokinase [13] and aldolase (D-fructose-1,6-biphosphate D-glyceraldehyde-3-phosphate lyase, EC 4.1.2.13) [14], from complexes with muscular contractile proteins. Accordingly we wondered whether this compartmentation of adenylosuccinate synthetase and glycolytic enzymes might economize the regulation of energy supply for muscle contraction from glycolysis. This also led us to speculate whether the three enzymes of the purine nucleotide cycle might show compartmentation in muscular contractile proteins in such a way that the regulation of ammoniagenesis took place efficiently.

Muscle AMP deaminase is a sticky contaminant of preparation during isolation of myosin or actomyosin [15]. Recently, Ashby et al. [16] demonstrated that AMP deaminase prepared from rabbit muscle was bound to rabbit muscle myosin in vitro. Therefore, to test our idea, we examined the location of AMP deaminase in rat muscle by in vitro binding experiments.

We found that the binding of rat muscle AMP deaminase to myosin differs from that of rabbit muscle AMP deaminase; that is, rat muscle AMP deaminase is bound to the light meromyosin portion of myosin molecule in a ratio of 1 mol of AMP deaminase to 3 mol of myosin.

Materials and Methods

Chemicals. Male Wistar strain rats, weighing approximately 200 g, were used throughout. Nucleotides, trypsin and trypsin inhibitor were purchased from Sigma Chemical Co. Phosphocellulose and DEAE-cellulose (DE-52) were from Whatman Biochemicals, Sepharose 6B, from Pharmacia. Freund's complete and incomplete adjuvants were products of Difco Laboratory. Other reagents were of analytical reagent grade.

Assays. AMP deaminase was assayed on the basis of colorimetric determination of ammonia liberated from AMP. The standard assay system contained enzyme solution, 4 mM AMP and 50 mM Tris-maleate buffer (pH 6.5) in a final

volume of 0.6 ml. Other enzymes were assayed according to the procedures of the following authors: adenylosuccinate synthetase, Ogawa et al. [9]; phosphofructokinase, Ling et al. [18]; adenylate kinase (EC 2.7.4.3), Oliver [19]; Ca^{2+}-activated myosin ATPase and Ca^{2+}, Mg^{2+}-activated actomyosin ATPase, Sugita et al. [20]. One unit of enzyme activity was defined as the amount catalyzing the formation of 1 μmol of product per min at 37°C. Protein concentration was determined by the method of Lowry et al. [22] with bovine serum albumin as the standard.

Preparation of enzymes. AMP deaminase from skeletal muscle was purified by a modification of the method of Smiley et al. [22], which was originally used for the purification of the rabbit muscle enzyme. Rat muscle extract (1.3 l) was applied to a phosphocellulose column (2 × 20 cm) and the column was washed first with 1 l of extraction buffer, and then with 1 l of 0.45 M KCl solution, adjusted to pH 7.0 with 1 M KH_2PO_4. The enzyme was then eluted from the column with a linear gradient of KCl (0.45 to 1.4 M) in 500 ml of extraction buffer containing 0.1 mM dithiothreitol. AMP deaminase was thus purified 1516-fold from rat muscle extract and its specific activity was 1274 units per mg protein. The purified enzyme gave one protein band on SDS-polyacrylamide gel electrophoresis. Sedimentation coefficient and the molecular weight of this enzyme were calculated to be 10.6 S and 243 000 ± 3000, respectively, by ultracentrifugal analysis. AMP deaminase from rat liver, kidney and cardiac muscle were partially purified by the method of Smith et al. [23]. Rat liver and kidney gave two peaks of AMP deaminase activity on DE-52 column chromatography. These enzyme fractions were designated type I and type II in order of elution. The characteristics and regulatory properties of type I and type II enzyme will be described elsewhere.

Adenylosuccinate synthetase from rat skeletal muscle was purified by the method of Ogawa et al. [9]. Phosphofructokinase was purified from rat skeletal muscle by ammonium sulfate fractionation, DE-52 column chromatography and Sephadex G-200 column chromatography. Purified adenylate kinase from skeletal muscle was kindly supplied by Dr. Tamura of our laboratory.

Preparation of rat muscle contractile proteins. Myosin was prepared from rat hind leg muscle by the method of Perry et al. [24] and further purified by column chromatography on Sepharose 6B to remove AMP deaminase and adenylate kinase.

Heavy meromyosin (H-meromyosin) and light meromyosin (L-meromyosin) were prepared with a trypsin digestion of myosin by the method of Yazawa et al. [25]. Light meromyosin was further purified by ethanol precipitation according to the method of Szent-Györgii et al. [26]. G-actin was prepared by the method of Mommaerts [27]. F-actin was obtained from G-actin in the presence of 0.1 M KCl. Actomyosin was prepared by mixing solutions of myosin and G-actin in 0.5 M KCl at physiological ratios, such as 2.7 mg of myosin and 1 mg of G-actin.

Binding experiments. Binding of AMP deaminase to F-actin was examined as described previously [12]. Experiments on the binding of AMP deaminase to myosin or actomyosin were carried out as follows. Purified AMP deaminase was mixed with myosin or actomyosin in 0.5 M KCl/50 mM Tris-HCl buffer (pH 7.0) and incubated at 37°C for 10 min. The reaction mixture was diluted with

50 mM Tris-HCl buffer (pH 7.0) to give a KCl concentration of 50 mM and centrifuged at 5000 × *g* for 10 min. The supernatant was discarded and the precipitate was washed with the same buffer. Bound enzyme was dissolved in 0.3 M KCl/50 mM Tris-HCl buffer (pH 7.0).

Preparation of antiserum against muscle AMP deaminase. AMP deaminase (1 mg of protein in 2 ml) from skeletal muscle was emulsified with 2 ml of Freund's complete adjuvant and injected subcutaneously into various regions of male rabbits. Two weeks later, a second dose was injected subcutaneously into other regions of the same animals, and 2 weeks later, a third injection was given in Freund's incompete adjuvant (1 mg of protein in 2 ml was emulsified with 2 ml of adjuvant). One week after the third injection, blood was collected from an ear vein and the serum was separated by centrifugation. The γ-globulin fraction was obtained by ammonium sulfate precipitation (0—30% saturation). The precipitated protein was dissolved in 20 mM Tris-HCl buffer/saline (pH 7.2) and dialyzed against the same buffer.

Results

Binding of AMP deaminase to muscle contractile protein

Rat skeletal muscle was homogenized with 3 vols. of 50 mM Tris-HCl buffer (pH 7.0) containing various amounts of KCl at 0°C; the homogenate was centrifuged and AMP deaminase activity in the supernatant was measured. As shown in Fig. 1, only 16% of the enzyme activity could be extracted with 50 mM Tris-HCl buffer without KCl, but the enzyme activity increased with increase in the KCl concentration in the buffer. Complete extraction was achieved with buffer containing KCl at concentration of more than 0.3 M. These results suggest that rat muscle AMP deaminase interacts with a muscle component, since the extraction of AMP deaminase from muscle homogenate resembles that of muscle contractile protein, especially myosin or actomyosin. Therefore, the

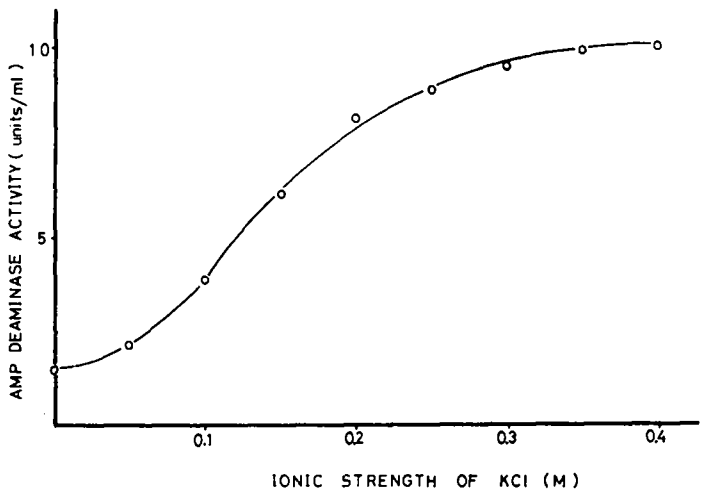

Fig. 1. Influence of ionic strength of the extraction of AMP deaminase from rat muscle. Enzyme activity in the supernatant was determined in the presence of 0.1 M KCl.

binding of AMP deaminase to muscle contractile proteins, such as myosin, actomyosin and F-actin, was examined. As summarized in Table I, AMP deaminase was almost completely precipitated on incubation with myosin or actomyosin, whereas only 24% was precipitated on incubation with F-actin. This suggests that AMP deaminase is mainly adsorbed to myosin in muscle cells.

Fig. 2 shows the effect of the concentration of AMP deaminase on its binding to a fixed amount of myosin in vitro. These experiments were performed with the precipitate solubilized in 0.3 M KCl/50 mM Tris-HCl (pH 7.0). Under these conditions, myosin would have no effect on AMP deaminase activity, since the two proteins are completely dissociated. The binding of AMP deaminase is defined as the enzyme activity coprecipitated with myosin. Scatchard plots showed that 0.38 mol of AMP deaminase was bound to 1 mol of myosin, or that 1 mol of AMP deaminase was bound to about 3 mol of myosin. The dissociation constant (K_d) was calculated to be 0.06 μM.

We then examined whether the binding to myosin was specific for muscle AMP deaminase. Adenylosuccinate synthetase, phosphofructokinase and adenylate kinase from rat skeletal muscle and AMP deaminase isozymes from rat liver (type I and II), kidney (type I and II) and cardiac muscle were all tested as possible binders. As indicated in Table II, these enzymes showed no interaction with myosin.

Influence of ionic strength and pH on the binding

Fig. 3 shows the effect of KCl concentration on dissociation of the myosin-AMP deaminase complex. The experiment was performed in the presence of sufficient myosin (almost 10-fold excess) to allow complete binding of AMP deaminase. As shown in the figure, the amount of precipitated complex decreased with increase in the KCl concentration. The supernatant in 0.3 M KCl was subjected to Sepharose 6B column chromatography to check whether it contained a complex of the two proteins. As shown in Fig. 4, this possibility was excluded, since AMP deaminase and myosin were eluted in completely separate positions and no complex could be detected. The same result was obtained with the supernatant in 0.15 M KCl/50 mM Tris-HCl buffer (pH 7.0). These findings indicate that the interaction of AMP deaminase and myosin

TABLE I

BINDING OF AMP DEAMINASE TO MUSCLE CONTRACTILE PROTEINS

Seven units of purified AMP deaminase were incubated with 1 mg sample of myosin and actomyosin in 0.5 M KCl 50 mM Tris-HCl buffer (pH 7.0) and F-actin in 50 mM Tris-HCl buffer (pH 7.0). The precipitate and supernatant were separated by centrifugation and the enzyme activity of each fraction was determined as described in the text.

Muscle protein	Percent recovery of AMP deaminase activity		
	Precipitate	Supernatant	Total
None	0	100	100
Myosin	98.4	3.4	101.8
Actomyosin	102.3	2.1	104.4
F-actin	24.0	78.7	102.7

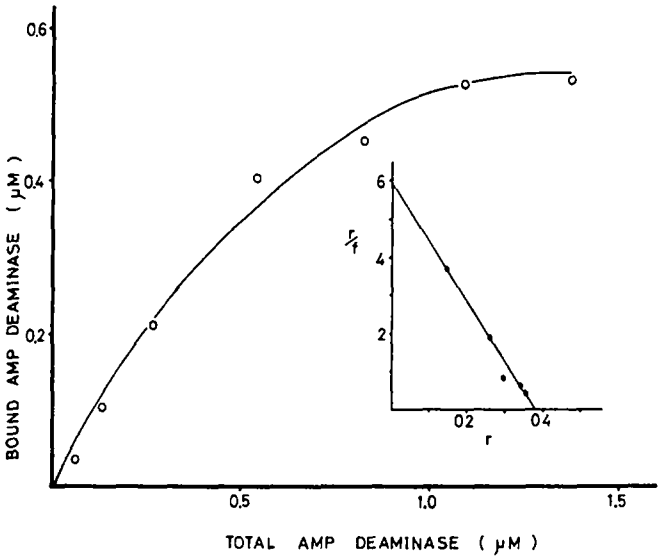

Fig. 2. Binding of AMP deaminase to myosin. Myosin (1.5 μmol in 0.5 M KCl/50 mM Tris-HCl buffer (pH 7.0) was mixed with various amounts of purified AMP deaminase (specific activity, 1274 units per mg protein) in the same buffer and incubated at 37°C for 10 min. The reaction mixture was diluted with 50 mM Tris-HCl buffer (pH 7.0) to give a KCl concentration of 50 mM and centrifuged at 5000 × *g* for 10 min. The supernatant was collected and the precipitate was washed with 0.5 ml of the same buffer. The supernatants were combined and the precipitate was dissolved in 0.3 M KCl/50 mM Tris-HCl buffer (pH 7.0). The inset shows a Scatchard plot of the data, in which *r* indicates the molar concentration of AMP deaminase bound per mol of myosin and *f* indicates the concentration of free AMP deaminase.

TABLE II

BINDING TO MYOSIN OF AMP DEAMINASE FROM RAT LIVER' KIDNEY AND CARDIAC MUSCLE AND ADENYLOSUCCINATE SYNTHETASE, PHOSPHOFRUCTOKINASE AND ADENYLATE KINASE FROM RAT SKELETAL MUSCLE

In this experiment, enzyme solution (liver type I, 0.95 unit; liver type II, 0.67 unit; kidney type I, 0.45 unit; kidney type II, 0.38 unit; cardiac muscle, 0.74 unit; skeletal muscle, 1.23 units; adenylosuccinate synthetase, 0.64 unit; phosphofructokinase, 0.76 unit; and adenylate kinase, 2.4 units) were incubated with myosin (1.72 mg) in 50 mM Tris-HCl buffer (pH 7.0). n.d., not determined.

Enzyme	Percent recovery of AMP deaminase activity		
	Precipitate	Supernatant	Total
AMP deaminase			
Skeletal muscle	99.3	0.2	99.5
Cardiac muscle	11.4	79.9	91.3
Liver type I	12.3	78.3	90.6
Liver type II	6.3	84.5	90.8
Kidney type I	9.2	88.2	97.4
Kidney type II	7.6	90.4	98.0
Adenylosuccinate synthetase	n.d.	97.4	n.d.
Phosphofructokinase	2.0	98.1	100.1
Adenylate kinase	n.d.	98.0	n.d.

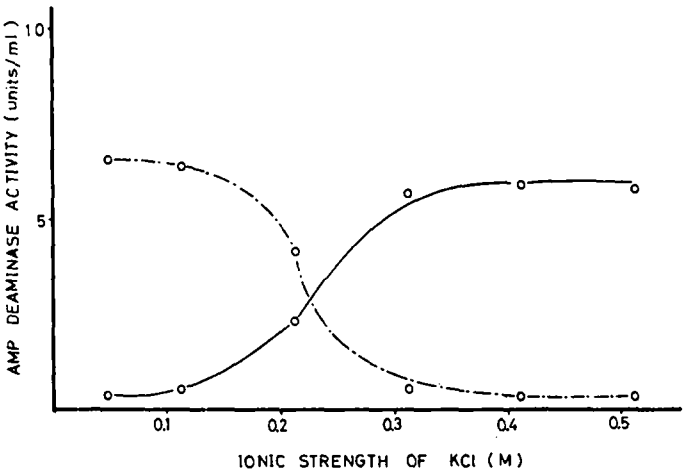

Fig. 3. Influence of ionic strength on the dissociation of myosin-bound AMP deaminase. The myosin-AMP deaminase complex was prepared by the incubating 7 units of AMP deaminase and 0.3 mg of myosin. o———o, enzyme activity in the supernatant; o— · — · —o, enzyme activity in the precipitate.

depends on the ionic strength of the environment and that AMP deaminase binds only to the fillamentous structure of myosin polymer formed at KCl concentrations of less than 0.3 M. The binding of AMP deaminase to myosin was not influenced by pH within the range of pH 6.5—7.5 (data not shown).

Binding of AMP deaminase to myosin subfragments

Myosin subfragments, H-meromyosin and L-meromyosin, were prepared to examine which part of the myosin molecule combined with AMP deaminase. In this experiment, purifed AMP deaminase was incubated with H-meromyosin

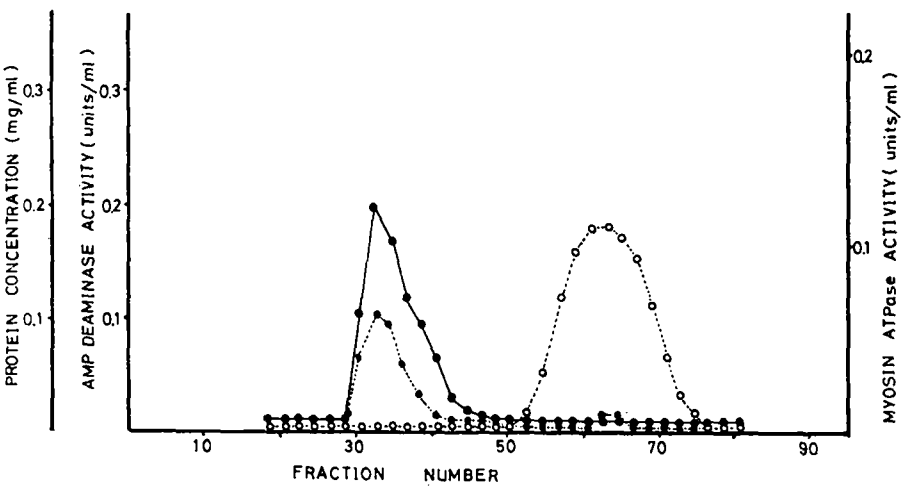

Fig. 4. Elution profile of AMP deaminase and myosin on Sepharose 6B column chromatography of the supernatant in 0.3 M KCl/50 mM Tris-HCl buffer. Fractions of 2 ml were collected. ●———●, myosin ATPase activity; o- - - - -o, AMP deaminase activity; ●- - - - -●, protein concentration.

(210 μg) in 50 mM Tris-HCl buffer (pH 7.0) or L-meromyosin (90 μg) in 0.5 M KCl/50 mM Tris-HCl buffer (pH 7.0) at 37°C for 10 min. In the experiment with H-meromyosin as an adsorbent, H-meromyosin plus AMP deaminase was precipitated by addition of F-actin and the precipitate was collected by centrifugation at 105 000 $\times g$ for 3 h. A correction was made for the activity at AMP deaminase precipitated nonspecifically with F-actin. 91.4% of the added AMP deaminase was precipitated on incubation with L-meromyosin, while only 26.7% was precipitated with H-meromyosin. Thus rat muscle AMP deaminase seems to bind mainly to the L-meromyosin portion of myosin molecule. This result is apparently different from that obtained by Ashby et al. [16], indicating that rabbit muscle AMP deaminase was bound to H-meromyosin and subfragment-2. Fig. 5A shows the results of Ouchterlony gel double diffusion of the antiserum against rat muscle AMP deaminase. The antiserum gave a single precipitin line against rat and mouse enzyme extracts and the two lines fused completely without spur formation. The antiserum also gave a single precipitin line against rabbit muscle enzymes extract, but this line formed a spur with the lines against rat and mouse extracts. In confirmation of these results, the AMP deaminase activities of rat and mouse muscle were strongly inhibited by the antiserum, but that of rabbit muscle was only partially inhibited (Fig. 5B). These results indicate that the antiserum reacts specifically with rat or mouse

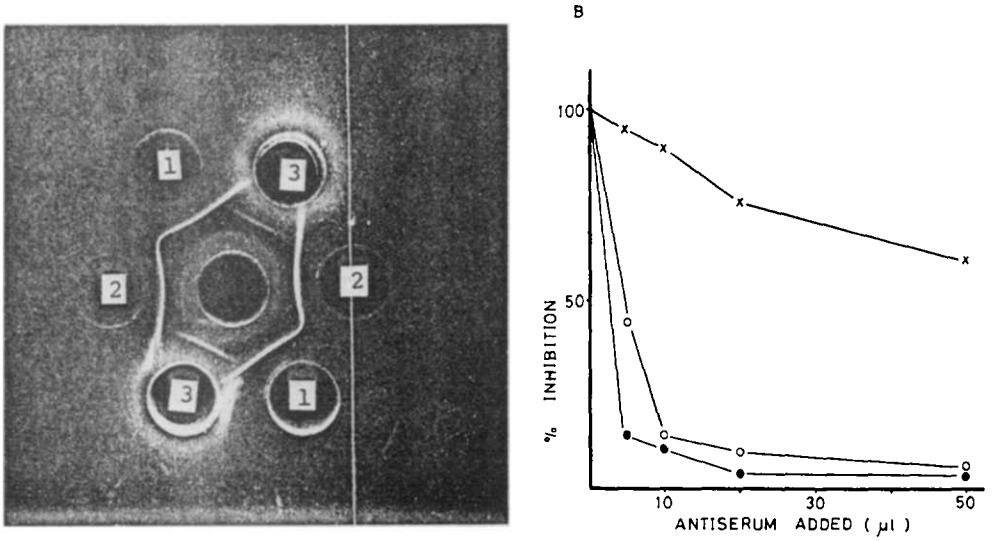

Fig. 5. Immunochemical analysis of muscle AMP deaminase with antiserum against purified AMP deaminase from rat skeletal muscle. A. Ouchterlony gel double diffusion test. The center well contained 100 μg of antiserum and well 1, 2 and 3 contained 100 μl of rat skeletal muscle enzyme (4.1 units), mouse skeletal muscle enzyme (3.4 units) and rabbit skeletal muscle enzyme (5.1 units), respectively. B. Inhibition of enzyme activity by antiserum. The reaction mixture contained 20 mM Tris-HCl buffer (pH 7.0), enzyme solution (rat muscle enzyme 2.1 units, mouse muscle enzyme 1.7 units and rabbit muscle enzyme 2.2 units, respectively) and various amount of antiserum in a total volume of 0.3 ml. The reaction mixture was incubated for 30 min at 37°C was left standing for 12 h at 4°C, and then centrifuged for 20 min at 3000 rev./min. Enzyne activity in the supernatant was determined. As a control, normal rabbit serum was added to the incubation mixture in place of antiserum. ●———●, rat muscle AMP deaminase; ○———○, mouse muscle AMP deaminase; X———X, rabbit muscle AMP deaminase.

muscle AMP deaminase, and that rabbit muscle AMP deaminase differs immunochemically from rat or mouse muscle AMP deaminase.

Discussion

In this work we have shown that in vitro both crude and purified rat muscle AMP deaminase associated reversibly with myosin, and that the enzyme has higher affinity for the light meromyosin portion than for the heavy meromyosin portion. We carried out the experiments of binding of AMP deaminase to myosin in 50 mM Tris-HCl buffer (pH 7.0) as well as in 0.5 M KCl/50 mM Tris-HCl buffer (pH 7.0), and found no difference in the binding capacity of myosin polymer to AMP deaminase in the two above buffers. These results indicate that the binding of AMP deaminase to myosin is not due to an artifact.

We have also shown that myosin does not interact with AMP deaminase isozymes from rat liver, kidney and cardiac muscle, all of which have been found to differ from the AMP deaminase in rat skeletal muscle in chromatographic and immunochemical properties (unpublished data). Moreover, the enzyme from rat skeletal muscle gave a single peak of activity on chromatography and electrophoresis. These findings indicate that the binding is specific for AMP deaminase of skeletal muscle. We have shown that AMP deaminase binds to myosin in a molar ratio of 1 : 3, and have calculated the dissociation constant as 0.06 μM. This high affintiy may explain why AMP deaminase is a sticky contaminant during purification of myosin or actomyosin.

Recently, Ashby et al. demonstrated by zonal centrifugation analysis in 0.15 M KCl solution that rabbit muscle AMP deaminase was bound to myosin, heavy meromyosin and subfragment 2 in a molar ratio, AMP deaminase to myosin, of 2 : 1 [16]. At a concentration of 0.15 M KCl, rat muscle AMP deaminase was almost completely bound to myosin (Fig. 3) and solubilized AMP deaminase and myosin were detected in separated position upon Sepharose 6B column chromatography and zonal centrifugation in a sucrose density gradient. These results indicate that the interaction of rat muscle AMP deaminase with myosin is different from that of rabbit muscle AMP deaminase. Since myosin molecule in different vertebrates are knwon to be the same [29], this difference is probably due to a difference in the properties of rat and rabbit AMP deaminase as shown in Fig. 5A.

What is the physiological meaning of this binding in rat muscle? As will be described in the following paper, we demonstrated that the activity of AMP deaminase from rat muscle was considerably enhanced by interaction of the enzyme with myosin or L-meromyosin, and also that myosin-bound AMP deaminase is more sensitive to regulation by nucleotides than is free AMP deaminase. On the other hand, we previously reported [12] that adenylosuccinate synthetase from rat muscle interacts with F-actin. In our laboratory, adenylsuccinase was shown to be a soluble enzyme, although it could not be purified because it is unstable. From this finding and the present results, it is suggested that the close approximation of myosin, to which AMP deaminase is bound, to actin, to which adenylosuccinate synthetase is bound by the sliding mechanism involved in muscle contraction, triggers off the cyclic operation of

the purine nucleotide cycle. This working hypothesis is consistent with the increase in ammoniagenesis during muscular contraction.

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