Interaction of AMP-aminohydrolase with Myosin and Its Subfragments*

BARRIE ASHBY AND CARI. FRIEDEN
From the Department of Biological Chemistry, Division of Biology and Biomedical Sciences, Washington University School of Medicine, St. Louis, Missouri 63110

We have shown that purified rabbit skeletal muscle AMP-aminohydrolase binds to rabbit muscle myosin, heavy meromyosin, and Subfragment 2 but does not bind to light meromyosin nor to Subfragment 1. The dissociation constant for binding to myosin was determined to be 0.14 μM. A new sedimentation boundary, presumably reflecting formation of a complex between AMP-aminohydrolase and heavy meromyosin or Subfragment 2, can be observed using the analytical ultracentrifuge. Binding of AMP-aminohydrolase to myosin, heavy meromyosin, or Subfragment 2 is abolished by phosphate (~10 mM), an inhibitor of AMP-aminohydrolase and phosphofructokinase or phosphocreatine kinase in the analytical ultracentrifuge. There was no indication of complex formation between AMP-aminohydrolase and myosin or Subfragment 2, can be observed using the analytical ultracentrifuge. Binding of AMP-aminohydrolase to myosin was determined to be 0.14 PM. A new sedimentation boundary, presumably reflecting formation of a complex between AMP-aminohydrolase and heavy meromyosin or Subfragment 2, can be observed using the analytical ultracentrifuge. Binding of AMP-aminohydrolase to myosin, heavy meromyosin, or Subfragment 2 is abolished by phosphate (~10 mM), an inhibitor of AMP-aminohydrolase and phosphofructokinase or phosphocreatine kinase in the analytical ultracentrifuge.

AMP-aminohydrolase, which utilizes AMP to produce IMP and ammonia, is present in many tissues but its level is particularly high in muscle (1). Although the activity of the enzyme appears to be affected by purine nucleotides binding to sites other than the active site (2), which suggests that the enzyme may have a regulatory function, its physiological role remains unclear. However, it is known that ammonia production in muscle is proportional to the work done (3) and that the actomyosin ATPase activity of different muscle types is directly related to the AMP-aminohydrolase activity as well as the phosphofructokinase activity (4, 5).

On the basis of such evidence, it has been suggested (6) that ammonia production may stimulate phosphofructokinase, the key enzyme in glycolysis, and thus exert regulatory control on the production of ATP for muscle contraction. Alternatively, the ADP produced from ATP during muscle contraction may lead to higher AMP levels via the adenylate kinase reaction. Increased ammonia production may then be a consequence of the action of the aminohydrolase on AMP.

AMP-aminohydrolase has been observed to be a persistent minor contaminant of myosin preparations (7, 8). In this paper, we show that purified AMP-aminohydrolase binds tightly to myosin, heavy meromyosin (HMM), and Subfragment 2 (S-2) but does not bind to light meromyosin (LMM) nor to Subfragment 1 (S-1). We also show that the binding appears to be specific in the sense that there are approximately 2 mol of enzyme bound per mol of myosin and that the interaction may be affected by the presence of certain ligands.

MATERIALS AND METHODS

AMP-aminohydrolase – AMP-aminohydrolase was prepared from rabbit muscle by the method of Smiley et al. (9) up to the stage of elution from cellulose phosphate. The protein was precipitated with an equal volume of saturated ammonium sulfate solution adjusted to pH 7.0 and the precipitate, collected by centrifugation, redissolved and dialyzed against 10 mM imidazole/HCl, 0.15 M KCl; 0.2 mM dithiothreitol, pH 7.0, and stored at a concentration of about 7 mg/ml.

Since the enzyme is much less soluble at low KCl concentrations, it was diluted from stock solution with buffer containing 1.0 M KCl to about 2.5 mg/ml before dialysis against 10 mM imidazole/HCl, 0.15 M KCl, pH 6.5, for use in analytical ultracentrifuge experiments. The protein concentration was determined from the absorbance at 280 nm using a molar extinction coefficient of 170 molar−1 cm−1 (10). Enzyme, prepared by this method, had a specific activity of 170 PM min−1 (mg of protein)−1 when assayed in 100 mM AMP, 10 mM imidazole/HCl, 0.15 M KCl, pH 6.5, at 25°C.

Myosin and Its Subfragments – Myosin was prepared by the method of Holtzer and Lowey (11). For some experiments, the myosin was purified by chromatography on DEAE-Sephadex (12) in order to remove contaminating AMP-aminohydrolase. Since this contamination was less than 2%, it did not significantly affect the kind of experiments reported here, and identical results were obtained using either preparation.

HMM and LMM were prepared by the method of Lowey and Cohen (13). LMM was further purified by precipitation with ethanol according to the method of Szent-Gyorgyi et al. (14). S-1 and S-2 were prepared by papain digestion of precipitated myosin and HMM, respectively, using iodoacetic acid to stop the reaction, as described by Lowey et al. (15). S-2 was further purified by ethanol precipitation (15).

Protein concentrations were determined using the following A280 values: 5.43 for myosin (16), 6.47 for HMM (17), 2.4 for LMM (14), 7.7 for S-1 (18), and 0.7 for S-2 (15). Molecular weights of 340,000 for HMM, 140,000 for LMM, 115,000 for S-1, and 82,000 for S-2, used to determine molar concentrations of the subfragments, were all taken from Lowey et al. (15). All other enzymes and reagents were obtained from Sigma Chemical Co.

Binding Experiments Using Myosin – Myosin in 10 mM imidazole/HCl, 0.5 M KCl, pH 6.5, was mixed with various amounts of AMP-aminohydrolase in the same buffer. The mixture was diluted with 10 mM imidazole/HCl; pH 6.5, to a KCl concentration of 50 mM, thereby completely precipitating the myosin, and centrifuged (5 min at 8,000 × g) in an Eppendorf centrifuge, operated at room temperature. Free AMP-aminohydrolase was calculated from the supernatant A280. The measurements were corrected for the loss of absorbance (5 to 10%) of control samples of AMP-aminohydrolase centrifuged in the absence of myosin. In the absence of AMP-aminohydrolase, all of the myosin

* This work was supported in part by United States Public Health Service Grant AM-13332.

The abbreviations used are: HMM, heavy meromyosin; LMM, light meromyosin; S-1, Subfragment 1; S-2, Subfragment 2.
was precipitated upon dilution to a KCl concentration of 50 mM. Molar binding ratios were estimated using molecular weights of 276,000 for AMP-aminohydrolase (19) and 480,000 for myosin (20).

**Sedimentation Velocity Experiments** — Sedimentation velocity experiments were performed at 20° and 52,000 rpm in a Beckman Spinco model E ultracentrifuge. The resulting schlieren patterns were measured on a Nikon two-dimensional comparator. The sedimentation coefficients given are 

### Results

The binding of AMP-aminohydrolase to myosin, performed as described above, is shown in Fig. 1 for two different myosin concentrations. Scatchard plots of these data (Fig. 1, inset) give a mean value of 2.5 mol of AMP-aminohydrolase bound per mol of myosin at the two concentrations, 0.2 mg/ml (0.44 μM) and 0.09 mg/ml (0.2 μM) of myosin. The calculated dissociation constant (Kd) was 0.14 μM. In a similar type of experiment, in which myosin concentration was varied from 0 to 0.25 mg/ml (0.55 μM) and AMP-aminohydrolase concentration kept constant (0.4 mg/ml, 0.15 μM), a stoichiometry of about 2 mol of AMP-aminohydrolase bound per mol of myosin was also obtained. Potassium phosphate at concentrations as low as 10 mM was effective in abolishing the interaction between the two proteins as measured by this method.

Using the same binding assay as described above (and in the absence of phosphate), it was found that the following rabbit muscle enzymes do not bind to myosin: adenylate kinase, phosphocreatine kinase, phosphofructokinase, lactate dehydrogenase, and aldolase. In addition, by substituting LMM for myosin in the binding assay, it was shown that AMP-aminohydrolase does not bind to LMM.

Sedimentation velocity experiments were then performed using AMP-aminohydrolase and purified HMM. Fig. 2a shows the sedimentation velocity patterns for AMP-aminohydrolase (upper pattern) and HMM individually (lower pattern). The \( s_{20,w} \) of 11.6 S for AMP-aminohydrolase (2.1 mg/ml) is identical to that obtained by Smiley et al. (9) and the \( s_{20,w} \) for 6.4 S for HMM (2.6 mg/ml) agrees well with its published value of 7.2 S (13). The result of mixing AMP-aminohydrolase and HMM in 10 mM imidazole/HCl, 0.15 M KCl, pH 6.5, at molar ratios of 1:1 or 2:1 is shown in Fig. 2b (upper and lower patterns, respectively). The upper pattern, obtained at a concentration of 7.7 μM for each protein (2.1 mg/ml of AMP-aminohydrolase and 2.6 mg/ml of HMM) shows two peaks. The slower sedimenting peak corresponds to HMM while the faster peak, which appears to represent a complex between the two proteins, has an \( s_{20,w} \) value of 19.3 S. The lower pattern, obtained using a 2:1 mixture of 7.7 μM AMP-aminohydrolase (2.1 mg/ml) and 3.85 μM HMM (1.3 mg/ml) shows essentially a single peak with a sedimentation coefficient of 20.1 S, tending to confirm the stoichiometry determined earlier using myosin.

The effect of potassium phosphate on the formation of the complex was examined by repeating the experiment in the presence of 2 mM and 5 mM potassium phosphate (Fig. 2c, upper and lower patterns, respectively). Phosphate at a concentration of 2 mM reduces the size of the fast moving peak whereas at 5 mM phosphate the fast moving peak totally disappears in agreement with the result obtained in the binding experiment with myosin. The effect of sulfate and arsenate was also examined by addition of 5 mM sodium sulfate or 5 mM sodium arsenate to the protein mixture (results not shown). Arsenate had a greater effect in reducing the size of the fast moving peak than sulfate although neither anion totally abolished the new boundary as did phosphate at the same concentration.

The concentration of KCl used in the ultracentrifuge experiments (0.15 M) represents close to an optimum condition for observation of the AMP-aminohydrolase-HMM complex in the absence of phosphate. In 0.1 M KCl, the complex aggregated and precipitated during the run whereas in 0.3 M KCl, the complex was partially dissociated and in 0.5 M KCl, it was completely dissociated. Experiments carried out using AMP-aminohydrolase and LMM in 0.15 M KCl, at which concentration LMM is only slightly soluble and was present in the ultracentrifuge cell at a concentration of about 0.3 mg/ml, showed no evidence of a boundary that could have represented a complex between the two proteins. Similar sedimentation velocity experiments involving mixtures of AMP-aminohydrolase (2.0 mg/ml) with phosphocreatine kinase (3.0 mg/ml) or phosphofructokinase (3.0 mg/ml) also showed no evidence of complex formation.

Analytical ultracentrifuge experiments were performed using AMP-aminohydrolase and S-1. Fig. 3a shows the sedimentation velocity pattern for AMP-aminohydrolase (upper pattern) compared to that of S-1 (lower pattern). The \( s_{20,w} \) of 12.2 S for AMP-aminohydrolase (1.7 mg/ml) is similar to the value determined above and the \( s_{20,w} \) of 5.7 S for S-1 (2.0 mg/ml) is almost identical to the value of 5.8 S published by Lowey et al. (15). The upper pattern in Fig. 3b shows the result of mixing AMP-aminohydrolase (1.7 mg/ml; 6.3 μM) with S-1 (2.0 mg/ml; 15 μM). Two peaks were obtained with \( s_{20,w} \) values of 12.2 S and 5.7 S corresponding to AMP-aminohydrolase and S-1, respectively. Addition of 5 mM potassium phosphate to a sample taken from the same mixture of the two proteins showed an identical pattern (Fig. 3b, lower pattern). Apparently no complex is formed between AMP-aminohydrolase and S-1.

Sedimentation velocity patterns of AMP-aminohydrolase and S-2 individually are presented in Fig. 4a (upper and lower patterns, respectively). AMP-aminohydrolase (1.2 mg/ml) had an \( s_{20,w} \) value of 11.9 S and S-2 (1.5 mg/ml) gave an \( s_{20,w} \) value of 6.2 S that compares favorably with the value of 2.7 S given by Lowey et al. (15). The result of mixing AMP-aminohydrolase (1.2 mg/ml; 4.5 μM) and S-2 (1.5 mg/ml; 24 μM) in 10 mM imidazole/HCl, 0.15 M KCl, pH 6.5, is shown in Fig. 4b (upper pattern).
**Interaction of AMP-deaminase with Myosin and Its Subfragments**

**Fig. 2 (top).** Sedimentation velocity patterns of AMP-aminohydrolase, HMM, and the complex between the two proteins. All experiments were performed at 20° in 10 mM imidazole/HCl, 0.15 M KCl; pH 6.5, at a speed of 52,000 rpm; and all photographs were taken 32 min after reaching that speed. The phase plate angle was 55°. a, AMP-aminohydrolase (2.1 mg/ml) (upper pattern) and HMM (2.6 mg/ml) (lower pattern); b, AMP-aminohydrolase (2.1 mg/ml), HMM (2.6 mg/ml) (upper pattern) and AMP-aminohydrolase (2.1 mg/ml), HMM (2.6 mg/ml), and 2 mM potassium phosphate (upper pattern); c, AMP-aminohydrolase (2.1 mg/ml), HMM (2.6 mg/ml), and 5 mM potassium phosphate (lower pattern). Sedimentation proceeds from left to right.

**Fig. 3 (left).** Sedimentation velocity patterns of AMP-aminohydrolase and S-1. Experiments were performed at 20° in 10 mM imidazole/HCl, 0.15 M KCl, pH 6.5, at a speed of 52,000 rpm; and the photographs were taken 32 min after reaching that speed. The phase plate angle was 55°. a, AMP-aminohydrolase (1.7 mg/ml) (upper pattern) and S-1 (2.0 mg/ml) (lower pattern); b, AMP-aminohydrolase (1.7 mg/ml), S-1 (2.0 mg/ml) (upper pattern) and AMP-aminohydrolase (1.7 mg/ml), S-1 (2.0 mg/ml), and 5 mM potassium phosphate (lower pattern). Sedimentation proceeds from left to right.

**Fig. 4 (right).** Sedimentation velocity patterns of AMP-aminohydrolase and S-2. Experiments were performed at 20° in 10 mM imidazole/HCl, 0.15 M KCl, pH 6.5, at a speed of 52,000 rpm; and the photographs were taken 32 min after reaching that speed. The phase plate angle was 55°. a, AMP-aminohydrolase (1.2 mg/ml) (upper pattern) and S-2 (1.5 mg/ml) (lower pattern); b, AMP-aminohydrolase (1.2 mg/ml), S-2 (1.5 mg/ml) (upper pattern) and AMP-aminohydrolase (1.2 mg/ml), S-2 (1.5 mg/ml), and 5 mM potassium phosphate (lower pattern). Sedimentation proceeds from left to right.

The two peaks present are a slow moving one with an $s_{20w}$ value of 2.9 S that represents S-2 and a fast moving peak of 18.6 S that represents a complex between S-2 and AMP-aminohydrolase. Addition of 5 mM potassium phosphate to an otherwise identical mixture of the two proteins resulted in the lower pattern of Fig. 4b in which the apparent complex is dissociated to give a peak of 2.9 S and another of 11.6 S representing S-2 and AMP-aminohydrolase, respectively.

**DISCUSSION**

It seems clear that AMP-aminohydrolase interacts with myosin to form a complex. The location of AMP-aminohydrolase binding is apparently the S-2 portion which, in terms of the physical structure of the myosin molecule described by Lowey et al. (15), corresponds to a highly α-helical Y-shaped section linking the fibrous tail (LMM) to the two globular heads (S-1). The specificity of the interaction is indicated by a number of observations. (a) No other muscle enzyme tested bound to myosin and hence little nonspecific trapping in the myosin gel occurred. (b) AMP-aminohydrolase did not interact with phosphocreatine kinase nor phosphofructokinase so that the interaction with myosin was probably not due to an inherent "stickiness" of the AMP-aminohydrolase molecule. (c) Distinct sedimentation boundaries were observed in the ultracentrifuge between AMP-aminohydrolase and HMM or S-2 at relatively high ionic strength (0.15 M KCl) that would be expected to disrupt weak, nonspecific electrostatic interactions. (d) AMP-aminohydrolase bound to S-2 but not LMM, both of which contain high proportions of α helix, so that binding was not determined by some exclusive property of α helical structures. (e) Inorganic phosphate disrupted the complex at relatively low concentrations (~5 mM) and phosphate is an inhibitor of AMP-aminohydrolase (21) with an inhibition constant of <1 mM. (f) The stoichiometry of the binding was close to 2 molecules of AMP-aminohydrolase per mol of myosin and the dissociation constant was 0.14 μM, indicating that myosin has a high affinity for AMP-aminohydrolase.

The observation of a stoichiometry of about 2 mol of AMP-aminohydrolase bound per mol of myosin, determined using the purified proteins, would be in conflict with the in vivo amounts of these proteins since there is considerably less AMP-aminohydrolase than myosin (estimated from the amount of enzyme in the crude extract). However, these results may be compared to the case of C-protein (22, 23) which is a myofibrillar protein of unknown function. Using purified proteins, Moos et al. (23) determined a stoichiometry of about 1 mol of C-protein bound per mol of myosin, yet C-protein represents only about 2% of the total protein of the intact myofibril (22). Optical diffraction patterns obtained from electron micrographs of light meromyosin paracrystals treated with C-protein (23) showed that C-protein forms a series of transverse stripes on the paracrystal with a longitudinal repeat of about 40 nm. Presumably there are constraints in the intact filaments determined by the underlying myosin assembly which...
may restrict the binding of proteins, including AMP-aminohy-
drase, to particular regions.

As stated earlier, the physiological role of AMP-aminohy-
drase is unknown. Tornheim and Lowenstein (6) have sug-
gested that the enzyme is involved in a purine nucleotide
cycle. They postulate that the role of this cycle may be to
regulate the activity of phosphofructokinase (and hence ATP
production by glycolysis) not only through its influence on the
relative concentrations of AMP, ADP, and ATP but also by the
production of ammonia which is an activator of phosphofructo-
kinase. The functional relationship between AMP-aminohy-
drase and myosin follows since the purine nucleotide cycle
exerts a form of regulation of ATP production for muscular
contraction.

Acknowledgments—We would like to thank Mr. Bruce Bar-
shop for the preparation of the S-1 and S-2 fragments and Mrs.
Helen Gilbert for excellent technical assistance.

REFERENCES
1. Lowenstein, J. M. (1972) Physiol. Rev. 52, 382-414
chem. Z. 188, 15-23
J. Physiol. 229, 422-426
6304-6314
Commun. 20, 422-426
Chem. 242, 2502-2506
2186
1377
12. Richards, E. G., Chung, C.-S., Menzel, D. B., and Olcott, H. S.
(1967) Biochemistry 6, 528-540
Mol. Biol. 2, 133-142
Mol. Biol. 42, 1-29
Acta 41, 401-421
Biol. Chem. 239, 2822-2829
Biol. Chem. 240, 2428-2436
1852
894-908
2265
Biol. 97, 1-9