Binding of Adenylyl Imidodiphosphate, an Analog of Adenosine Triphosphate, to Myosin and Heavy Meromyosin*

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SUMMARY

The binding of adenylyl imidodiphosphate (App(NH)p), an analog of ATP with an imido group (—NH—) in place of the oxygen bridge between the β and γ phospholyl groups, was studied. Binding studies were performed at 23–25° in 0.020 M Tris-HCl, 1.00 mM MgSO₄, and 0.100 M NaCl at pH 7.4. The dissociation constant of App(NH)p with myosin, 0.400 ± 0.040 μM, is identical with the previously reported Kₐ value for ATP hydrolysis, 0.41 μM (SCHLISELFELD, L., AND RÅNSTRÖM, M. (1968) Biochemistry 7, 3206–3213). The dissociation constant of App(NH)p with heavy meromyosin, 0.393 ± 0.104 μM, is nearly identical with the Kₐ value for the hydrolysis of ATP, 0.164 ± 0.033 μM. The low salt-soluble heavy meromyosin has a maximum binding value of 1.18 ± 0.04 moles of App(NH)p per 500,000 g of protein. The low salt-soluble heavy meromyosin has a maximum binding value of 2.13 ± 0.08 moles of App(NH)p per 350,000 g of protein. This indicates that the aggregation of myosin molecules at low salt concentration causes a steric hindrance for 41% of the nucleotide binding sites.

Adenylyl imidodiphosphate is an analog of ATP with an imido group (—NH—) in place of the oxygen bridge between the β and γ phospholyl groups. This analog has been made by an organic synthesis procedure of Yount et al. (1) and by an enzyme-catalyzed reaction procedure of Rodbell et al. (2). App(NH)p is not hydrolyzed by myosin, heavy meromyosin, or subfragment 1 (EC 3.6.1.3, ATP-phosphohydrolase) (3, 4). The analog is the most potent known competitive inhibitor of the ATPase activities of myosin, heavy meromyosin, and subfragment 1 (3, 4), a study of the binding of this analog to the contractile proteins should be very valuable. Most importantly, the binding studies can be carried out at low salt concentration, which cannot be done with ATP due to the ATPase activity of myosin. This report will show that myosin has one App(NH)p binding site per molecule and heavy meromyosin has two App(NH)p binding sites per molecule at 0.10 M NaCl and pH 7.4. The dissociation constants of App(NH)p with both proteins are equal to their Kₐ values for ATP hydrolysis. Portions of these studies have been described in preliminary communications (10, 11).

EXPERIMENTAL PROCEDURE

Materials—The following materials were purchased: ATP and AMP from P-L Biochemicals, Inc., [8-3H]AMP from Schwarz-Mann, [γ-32P]ATP and nonradioactive App(NH)p from ICN, isopropyl acetate, 99% pure, from Aldrich Chemical Co., phosphocellulose from Bio-Rad, Whatman No. DE81 paper from Brinkmann Instruments, Inc., and Nessler's reagent from Standard Scientific Supply Co. All other reagents were the best available analytical grades. The commercial [γ-32P]ATP was mixed with 5 moles of nonradioactive ATP, neutralized with 2 M Tris to pH 8 and diluted with sterilized distilled water to 5.00 ml. The radioactive solution was standardized by measuring its absorbance at 259 nm (12). The radioactive ATP contained 1.04% of its total radioactivity as 32P; in paper chromatography system A (see Table I) 98.5% of the radioactivity migrated with ATP. Myosin was prepared from the back and hind leg skeletal muscles of a rabbit by the procedure of Rånström and Oppenheimer (13). The myosin was further purified by passage through a column of phosphocellulose equilibrated in 0.040 M Tris-HCl and 0.40 M KCl at pH 7.8 and 5° as described by Harris and Sueter (14). The final protein solution in 0.60 M KCl was centrifuged at 92,000 X g for 2 hours to remove insoluble material. Heavy meromyosin was prepared by tryptic digestion of myosin followed by chromatography on DEAE-cellulose (15).

Synthesis of [γ-32P]ATP/ND—Yount et al. (1) synthesized nonradioactive App(NH)p by a chemical procedure that required large amounts of reactants. This did not appear to be a satisfac-
Ammonia was determined with a Conway diffusion cell followed by a reaction with Nessler’s reagent (20). The Conway diffusion cell was necessary because the analog was treated with the acid hydrolysis of App(NH)p react with Nessler’s reagent to form a precipitate that interferes with a direct ammonia analysis. Into the center well of each Conway diffusion cell was placed 1.00 ml of 0.10 N H$_2$SO$_4$. In the perimeter well was placed at one end 0.05 ml of 19.4 N NaOH and at the opposite end 2.00 ml of sample or 2.00 ml of distilled water. Each cell was covered with a glass plate to give an air-tight seal. The cells were then tipped sufficiently to cause the solutions in the perimeter wells to mix thoroughly. The cells were left at room temperature for 12 hours to permit the quantitative transfer of ammonia from the perimeter wells to the center wells. The glass plate covers were removed, and the solution in center well plus three 1.0-ml distilled water washes of each center well were transferred to 10 ml graduated centrifuge tubes. Each solution was diluted with distilled water to 4.50 ml and then mixed with 0.50 ml of Nessler’s reagent. After 15 min at room temperature the solutions were read in a spectrophotometer at 460 nm. A standard curve for ammonia was prepared with A.C.S. reagent grade (NH$_4$)$_2$SO$_4$.

<table>
<thead>
<tr>
<th>Nucleotide</th>
<th>System A</th>
<th>System B</th>
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<tbody>
<tr>
<td>ATP</td>
<td>0.18</td>
<td>0.14</td>
</tr>
<tr>
<td>ADP</td>
<td>0.44</td>
<td>0.36</td>
</tr>
<tr>
<td>AMP</td>
<td>0.76</td>
<td>0.54</td>
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<tr>
<td>App(NH)p</td>
<td>0.26</td>
<td>0.26</td>
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<table>
<thead>
<tr>
<th>Nucleotide</th>
<th>r$_D$</th>
<th><em>r</em>{max}</th>
</tr>
</thead>
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<tr>
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<td>0.26</td>
<td>0.26</td>
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Equation 1. [App(NH)p]$_i$ = $-K_D + _r_{max} [App(NH)p]/r$

The binding of [8-3H]App(NH)p to heavy meromyosin at low salt concentration was determined by the gel filtration procedure of Hummel and Dreyer (22) as described for the binding of [γ-3²P]ATP to myosin and heavy meromyosin (22). All of these experiments were carried out at room temperature (23-25°C) in 0.020 M Tris-HCl, 1.000 mM MgSO$_4$, 0.100 mM NaCl, and varying concentrations of [8-3H]App(NH)p at pH 7.4. Duplicate tubes for each analog concentration were prepared. One set of tubes contained 0.855 mg per ml of myosin; the second set of tubes did not have any protein. The tubes were stopped, shaken thoroughly to disperse the protein, incubated at 25°C for 10 min, and then shaken again to disperse the protein. Next the tubes were centrifuged in the x/40 head of the refrigerated Beckman ultracentrifuge model L at 92,000 X g for 45 min. The centrifuge was stopped with the automatic brake off to minimize disturbances in the supernatants. Next 0.50 ml of each solution was taken from just below the meniscus and counted for radioactivity. The free App(NH)p concentration was calculated from the radioactivity in the supernatant above the myosin protein precipitate. The protein-bound nucleotide was determined from the difference between the radioactivity in the solutions without and with the myosin protein precipitate of the initially duplicate analog concentrations.

The binding of [8-3H]App(NH)p to heavy meromyosin at low salt concentration was determined by the gel filtration procedure of Hummel and Dreyer (22) as described for the binding of [γ-3²P]ATP to myosin and heavy meromyosin (22). All of these experiments were carried out at room temperature (23-25°C) in 0.020 M Tris-HCl, 1.000 mM MgSO$_4$, 0.100 mM NaCl, and varying concentrations of [8-3H]App(NH)p at pH 7.4. The results of these binding studies were plotted according to Equation 1. [App(NH)p]$_i$ is the free nucleotide concentration, [App(NH)p]$_i$ is the binding value for a given nucleotide concentration, $K_D$ is the equilibrium dissociation constant, and _r_{max} is the maximum binding value. Linear regression analysis was employed to determine the correlation coefficient for each line and to obtain the average values for _K_D and _r_{max} plus their standard errors. ATPase Activity Buffered solutions of [γ-3²P]ATP were pre incubated at 25°C for 5 min. Then a solution of heavy meromyosin

No significant difference was observed for the concentration of [8-3H]App(NH)p in the myosin-free solution before and after centrifugation. Therefore, the analog did not sediment during the high speed centrifugation step.

The 1,1’ carbonyldiimidazolo, tributylamine, distilled triethylamine, and other reagents used in the synthesis of [8-3H]App(NH)p were provided by Dr. Alexander J. Murphy, College of the Pacific, San Francisco, Calif.
RESULTS

Characterization of [8-3H]App(NH)p—The P1 and ammonia in the [8-3H]App(NH)p solution were determined before acid hydrolysis and after a 10-min hydrolysis in 1.0 N H2SO4 at 105°. No significant P1 or ammonia is found in the analog solution before hydrolysis. The purified [8-3H]App(NH)p has a molar ratio of acid-labile P1 to acid-labile ammonia to total adenine of 1.83:0.06:1.00. This is essentially equal to the analysis reported by Yount et al. (1).

The purity of the analog was checked by chromatography on Whatman DEAE-paper, System A, and on Brinkmann precoated sheets of CEL 300 PEI, System B. In System A only one ultraviolet light-absorbing spot was found, and it contained 95.9% of the total radioactivity placed on the origin. This spot had an Rf value of 0.27, which is the same as the value given in System A by App(NH)p (see Table I). The remaining 4.1% radioactivity had an Rf value of 0.57. In System B there was only one ultraviolet light-absorbing spot, and it contained 97.0% of the total radioactivity placed on the origin. The Rf value of this spot was 0.27, which is the same as the value given by App(NH)p in System B (see Table I). The remaining 3.0% of the total radioactivity had an Rf value of 0.72. The concentration of the analog is the adenine concentration times the fraction bound per mole of myosin. Myosin has been shown to have two nucleotide-binding sites per molecule (23, 27-30). This suggests that at low salt concentration the aggregation of myosin causes a steric hindrance to the binding by 41% of the nucleotide-binding sites on the protein.

Binding of [8-3H]APP(NH)p to Heavy Meromyosin—The results of a typical binding study of 4.53 μM [8-3H]App(NH)p to 5.59 mg of heavy meromyosin on a column of Sephadex G-50 is shown in Fig. 2. A peak of radioactivity appears in the effluent simultaneously with 96.5% of the protein placed on the column. After a brief return to the base radioactivity level a trough of radioactivity appears. The radioactivity peak corresponds to 27.0 nmoles of [8-3H]App(NH)p, and the trough corresponds to 28.0 nmoles of [8-3H]App(NH)p. The value for r is calculated from the average of the nucleotide in the peak and the trough divided by the protein placed on the column. For the experiment in Fig. 2 the value for r is 4.92 nmoles of App(NH)p bound per mg of protein.

A graphical presentation of the binding data for App(NH)p with heavy meromyosin is shown in Fig. 3. A straight line is obtained for free App(NH)p concentrations ranging from 0.226 to 4.53 μM with a correlation coefficient of 0.990. The KD value is 0.393 μM. The rmax value is 5.80 nmoles of App(NH)p bound per mg of protein or 2.13 moles of App(NH)p bound per mole of heavy meromyosin.

The Km Value for Hydrolysis of ATP by Heavy Meromyosin—Fig. 4 presents a plot of [ATP] versus [ATP]/v for the Mg2+-ATPase activity of heavy meromyosin in 0.100 M NaCl at 7.4 and 25°. A straight line plot is obtained in the ATP concentration range of 0.050 to 2.00 μM. A linear regression analysis yields a correlation coefficient of 0.066 for this straight line. The Km value obtained from the ordinate intercept is 0.164 μM; the
Fig. 2. Gel filtration binding study of 4.53 μM [3-H]App(NH)p to heavy meromyosin. A column (1.5 x 20 cm) of Sephadex G-50 fine was equilibrated in 0.020 M Tris HCl, 1.60 mM MgSO₄, 0.100 M NaCl, and 4.53 μM [3-H]App(NH)p at pH 7.4 and room temperature. Then 1.00 ml of 5.59 mg per ml of heavy meromyosin in 0.020 M Tris-HCl, 1.00 mM MgSO₄, 0.100 M NaCl, and 4.53 μM [3-H] App(NH)p at pH 7.4 and room temperature was placed on the column. The protein was washed through the column with the protein-free, 4.53 μM [3-H]App(NH)p equilibration solution. The effluent was collected by hand at 1.3 ml per min in 12-ml graduated centrifuge tubes, and each tube had at least 2.15 ml of solution. The solution volume in each tube was noted and then 0.50 ml of each solution was counted for radioactivity. The solutions in the first 10 tubes were assayed for protein. The protein peak (tubes 5 and 6) contained 5.39 mg of protein. The radioactivity peak corresponds to 27.0 nmoles of App(NH)p; the radioactivity trough corresponds to 28.0 nmoles of App(NH)p.

Fig. 3. Binding of [3-H]App(NH)p to heavy meromyosin. The V₅₀ value given by the slope of the straight line is 11 nmoles per min per mg of heavy meromyosin. This K₅₀ value is within experimental error equal to the Kᵦ value of App(NH)p for heavy meromyosin.

DISCUSSION

Table II summarizes the constants and their standard errors obtained in these studies. For myosin there are no significant differences between the Kᵦ value for binding App(NH)p (0.400 μM), the previously reported Kᵦ value for the binding of ATP (0.84 ± 0.25 μM) (23), and the previously reported Kᵦ value for the hydrolysis of ATP (0.41 μM) (23). For heavy meromyosin there are no significant differences between the Kᵦ value for binding App(NH)p (0.303 μM), the Kᵦ value for the hydrolysis of ATP (0.164 μM), and the previously reported Kᵦ value for the binding of ATP (0.37 ± 0.08 μM) (23). The Kᵦ value for heavy meromyosin reported here is less than the previously reported value of 0.65 μM (23). This difference is due to random experimental error in the earlier Kᵦ determination. Yount et al. (3) have recently reported a Kᵦ value for the Mg₂⁺ATPase activity of heavy meromyosin of 0.11 μM. Lynn and Taylor (31) have reported that the Mg₂⁺ATPase activity of myosin yields a nonlinear Scatchard plot and a Kᵦ value of 0.05 μM.

The high correlation coefficient values obtained in these studies for the binding of App(NH)p and for the ATPase activity, 0.990 to 0.996, shows that no curvature occurs in these studies. Furthermore, the 95% confidence interval for the Kᵦ value, from 0.086 μM through 0.242 μM, is greater than the Kᵦ value reported by Lynn and Taylor (31). One possible explanation for these differences could be the differences in reaction pH, which is 7.4 in these studies and is 8.0 in the studies of Lynn and Taylor.

<table>
<thead>
<tr>
<th>Constant</th>
<th>Myosin</th>
<th>Heavy meromyosin</th>
</tr>
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<tbody>
<tr>
<td>Kᵦ, μM App(NH)p</td>
<td>0.400 ± 0.040</td>
<td>0.303 ± 0.104</td>
</tr>
<tr>
<td>Kᵦ, μM ATP</td>
<td>0.41*</td>
<td>0.164 ± 0.030</td>
</tr>
<tr>
<td>rₘₐₓ, moles of App(NH)p per mole of protein</td>
<td>1.18 ± 0.04</td>
<td>2.13 ± 0.08</td>
</tr>
</tbody>
</table>

* This Kᵦ value for myosin in 0.025 M and 1.52 M NaCl at pH 7.4 and 25° was reported previously (23).
Therefore, it is concluded that the dissociation constants for the binding of App(NH)p and ATP to either myosin or heavy meromyosin are identical.

The r_max value reported here for myosin at low salt concentration, 1.18 moles of App(NH)p bound per mole of protein, is significantly less than the r_max for heavy meromyosin under identical conditions. 2.13 moles of App(NH)p bound per mole of protein. Because of the great quenching of tritium radioactivity by salt, it has been impossible to obtain reliable binding data of [8-^H]App(NH)p for myosin dissolved in concentrated salt solution. However, myosin solubilized at high salt concentrations has two nucleotide-binding sites per protein molecule (23, 27–30). This indicates that aggregation of myosin at low salt concentration causes a steric hindrance at about 41 % of the nucleotide-binding sites. This suggests that under physiological conditions not all of the ATP-binding sites and F-actin-binding sites are available because of the insoluble condition of the myosin fibers. Chaplain and Tregear (32), using the water bug flight muscle, have reported one cross-bridge per 3 molecules of myosin. Recently Reedy et al. (33), using blowfly flight muscle, have reported one cross-bridge per 5 or 6 molecules of myosin. Though these studies were carried out on the flight muscles of insects, they suggest that similar results will be obtained with mammalian muscles.

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