The Binding of Inorganic Phosphate to Myosin in the Presence of Adenosine Triphosphate*

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It has been repeatedly shown that myosin or actomyosin (1, 2) contain small amounts of phosphate, in less than 1 mole per mole ratio. Recently Brahms and his associates reported that the amount of bound phosphate in frog myosin (3) or rabbit H-meromyosin (4), defined as the amount of inorganic phosphorus found in the isoelectric or ammonium sulfate precipitate of these proteins, increases during the splitting of adenosine triphosphate, reaches a maximum, and returns to its original value when all the adenosine triphosphate has been split. They suggested that the increase in bound phosphate is due to the presence of a phosphorylated protein intermediate.

According to Drabikowski (5), P, added to myosin and other proteins appears to be bound when the proteins are precipitated. In view of this fact it appeared of interest to reinvestigate the relationship of P-binding and ATPase activity with the well characterized myosin of the rabbit; it also seemed that by using P32 a clearer answer might be obtained to the question whether bound phosphate originates in ATP. Our results confirm those of Brahms et al. (3, 4) as regards the apparent P-binding during the course of the ATPase reaction. We found, however, that this can be ascribed to the binding of P1, which is enhanced by the presence of ATP. The isotope experiments strongly suggest that the P found in the precipitated myosin originates in P1, and is not directly derived from ATP.

EXPERIMENTAL PROCEDURE

Rabbit myosin, actin, and tryptic meromyosins were prepared according to the usual procedure in this laboratory (6). The determination of the bound P was carried out as follows: Myosin was precipitated at 0° by adding 5.0 ml of 0.2 M acetate buffer, pH 4.0; the precipitate was centrifuged at 18,000 × g for 20 minutes and washed three times with 5.0 ml of 0.3 M KCl-0.1 M acetate buffer solution, and finally extracted with 2 ml of 10% trichloroacetic acid (3). When comparisons with meromyosins were made (NH4)2SO4 was added to 35% saturation to precipitate the proteins, and the precipitates were then washed with 55% saturated (NH4)2SO4 (4). P determinations were carried out on the trichloroacetic acid supernatant with the use of the method described by Horwitt (7) or Marsh (8).

The ATPase reaction was carried out in the following system: 0.1 M KCl, 0.02 M Tris buffer, pH 7.4; 3 mM CaCl2; or 2 to 20 mM MgCl2 and 1 to 10 mM ATP in a total volume of 5.0 ml. The reaction was stopped by precipitating the protein as described above, the precipitate subjected to the bound P1 analyses, and the P1 liberated determined in the protein-free supernatant by the Fiske-SubbaRow method (9).

For radioactivity measurements samples were placed in stainless steel cups and counted with an end window Geiger counter.

Crystalline disodium ATP was obtained from Pabst Laboratories, Milwaukee, and p-chloromercuribenzoate from Sigma Chemical Company, St. Louis.

RESULTS

As the cleavage of ATP by rabbit myosin proceeds the phosphate found in the precipitated myosin increases until about 70% of the ATP is cleaved, and then gradually decreases, reaching a final value when all the ATP is hydrolyzed that is still somewhat higher than the initial one (Fig. 1). This pattern is quite similar to that found for frog myosin (3) and rabbit H-meromyosin (4). However, this phosphate-binding cannot be directly correlated with the ATPase activity. Considerable phosphate-binding took place even when the ATPase activity was reduced by treatment with p-chloromercuribenzoate or CuSO4 or by heat inactivation, if P1 was added to the reaction mixture (Table I). Table I also shows that even when no ATP cleavage takes place the presence of ATP increases the binding of P1 to myosin. The effect of ATP on the P1-binding showed a concentration dependence, the maximum being reached at about 10 mM ATP (Fig. 2). Among the tested nucleotides ATP and ADP were the only effective ones in increasing the P1-binding, but ADP was less effective than ATP (Table II).

The increase in P-binding on addition of ATP was also observed with both meromyosins. The P-binding to H-meromyosin was increased 3.5-fold, that to L-meromyosin 2.7-fold. These values are in the same range as those for myosin, viz., 2 to 4. No increase was found in the case of actin.

The remote possibility remained that the P-binding in the presence of ATP, even when there was no ATPase activity, might represent a direct transfer of P from ATP. If the bound phosphate came directly from ATP its specific activity should, in the presence of added P32 be lower than that of the total P. As shown in Table III no difference between these specific activities was brought about by the presence of ATP.

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Fig. 1. Binding of inorganic phosphate to myosin during the cleavage of ATP. These experiments were carried out in the presence of 3 mM CaCl₂ and 3.5 mM ATP; myosin concentration, 4 mg per ml. Other details of ATPase assay and of the determination of bound P are described under "Experimental Procedure." Left ordinate: liberation of P. Right ordinate: bound phosphate. O, Liberation of P; ⬤, binding of P. Abscissa: time of incubation.

Table I
Effect of inhibition of ATPase activity on phosphate-binding of myosin

Conditions as described in the legend of Fig. 1, but Mg⁺⁺ as indicated in the table, and, in the binding experiments, each tube contained 20 μmoles of P. Incubation was for 10 minutes. The details of the determination of P₁ liberation and of the bound P are described under "Experimental Procedure."* 

<table>
<thead>
<tr>
<th>Additions</th>
<th>Moles of P₁ per 10⁶ g of myosin</th>
<th>P₁ liberation* μmoles</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>1.5</td>
<td>2.8 15.3</td>
</tr>
<tr>
<td>20 μmoles of Mg⁺⁺</td>
<td>2.5</td>
<td>4.3 1.3</td>
</tr>
<tr>
<td>20 μmoles of Mg⁺⁺</td>
<td>0.7</td>
<td>2.9 0.0</td>
</tr>
<tr>
<td>20 μmoles of Mg⁺⁺ + 5 μmoles of p-chloromercuribenzoate</td>
<td>1.4</td>
<td>3.1 0.1</td>
</tr>
<tr>
<td>20 μmoles of Mg⁺⁺ + 10 μmoles of CuSO₄</td>
<td>1.7</td>
<td>2.5 0.0</td>
</tr>
</tbody>
</table>

* The ATPase activity was measured in the absence of added P₁.
† Myosin preheated for 5 minutes at 60°.

Fig. 2. Effect of ATP concentration on the binding of inorganic phosphate to myosin. Each tube contained 0.6 M KCl, 20 mM MgCl₂, 0.02 M Tris buffer, 20 mg of myosin, 4 mM P₁, and ATP as indicated on the abscissa, in a total volume of 5.0 ml. The mixture was incubated for 5 minutes at 23° and pH 7.4. For details of the determination of bound P see "Experimental Procedure."

Table II
Effect of several nucleotides on phosphate-binding of myosin

Each tube contained 0.6 M KCl, 0.02 M MgCl₂, 0.02 M Tris buffer, and 50 mg of myosin, in a total volume of 5 ml. P⁻¹ with a total radioactivity of 1.47 × 10⁶ c.p.m. was added. Other additions as indicated in the table. The mixture was incubated for 20 minutes at pH 7.4 and 23°. At the end of the incubation period the total and bound P and the radioactivity were determined as described under "Experimental Procedure."

<table>
<thead>
<tr>
<th>Nucleotide</th>
<th>Moles of bound P per 10⁶ g of myosin</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>1.2</td>
</tr>
<tr>
<td>IDP</td>
<td>2.0</td>
</tr>
<tr>
<td>ITP</td>
<td>1.5</td>
</tr>
<tr>
<td>AMP</td>
<td>1.5</td>
</tr>
<tr>
<td>ADP</td>
<td>3.1</td>
</tr>
<tr>
<td>ATP</td>
<td>6.0</td>
</tr>
</tbody>
</table>

Table III
Binding of P⁻¹ to myosin

Each tube contained 0.6 M KCl, 0.02 M MgCl₂, 0.02 M Tris buffer, and 50 mg of myosin, in a total volume of 5 ml. P⁻¹ with a total radioactivity of 1.47 × 10⁶ c.p.m. was added. Other additions as indicated in the table. The mixture was incubated for 20 minutes at pH 7.4 and 23°. At the end of the incubation period the total and bound P and the radioactivity were determined as described under "Experimental Procedure."

<table>
<thead>
<tr>
<th>Additions</th>
<th>20 μmoles of P₁</th>
<th>40 μmoles of ATP</th>
<th>40 μmoles of ATP plus 20 μmoles of P₁</th>
</tr>
</thead>
<tbody>
<tr>
<td>P₁ in reaction mixture</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total amount (μmoles)</td>
<td>19.0</td>
<td>8.0</td>
<td>27.4</td>
</tr>
<tr>
<td>Specific activity (c.p.m. × 10⁴ per μmole)</td>
<td>0.8</td>
<td>1.8</td>
<td>0.54</td>
</tr>
</tbody>
</table>

| Bound P₁ |                |                 |                                      |
| Amount (moles per 10⁶ g of protein) | 1.6             | 1.0             | 3.4                                 |
| Specific activity (c.p.m. × 10⁴ per μmole) | 1.2             | 1.6             | 0.50                                 |

DISCUSSION

These experiments clearly show that the increase in the amount of P₁ found in a washed myosin or meromyosin precipitate during the hydrolysis of ATP can be accounted for in terms of P₁-binding which is enhanced by the presence of ATP. The same amount of bound P is found when P₁ is added in the presence of ATP, in a concentration corresponding to that produced during ATP hydrolysis but the splitting is actually prevented by Cu⁺⁺ or mercurials. The transient increase of P₁-binding during the ATPase reaction can also be explained on this view; as P₁ is produced and ATP is still present in sufficient amounts the binding increases, but as the ATP is depleted the P₁-binding decreases again. That the final value is somewhat higher than the initial one can be ascribed to the weaker effect of ADP on P₁-binding (cf. Table II).

The ATP-enhanced P₁-binding is somewhat inhibited by p-chloromercuribenzoate or CuSO₄. This might be related to the observation of Brahms and Kakol that an increase in bound P is correlated to a decrease in the SH content of myosin (3),
and suggests the involvement of SH groups in this type of Pi-binding. Also, this type of Pi-binding might be closely related to the previous finding of Buchthal et al. that ATP increases Pi uptake of denatured actomyosin (1).

Experiments with P32 do not suggest a direct transfer from ATP, even if not related to hydrolysis, as the basis for the increased Pi in washed myosin precipitates. This ATP-enhanced Pi-binding was also observed with L-meromyosin devoid of ATPase activity. However, it is to be noted that the Pi-binding of serum albumin (cf. 5) and actin was not enhanced by ATP.

It would appear that experiments of this type cannot contribute to the problem of the participation of a phosphorylated protein intermediate in the ATPase reaction, the existence of which is suggested by the recent findings of Levy et al. showing O18 exchange between H2O18 and the phosphate formed in the hydrolysis of ATP by myosin and actomyosin (10).

SUMMARY

The binding of phosphate to myosin in the course of the cleavage of adenosine triphosphate can be ascribed to the binding of inorganic phosphate enhanced by adenosine triphosphate. Experiments with the use of P32-labeled inorganic phosphate suggest that the bound phosphate does not originate in adenosine triphosphate and is thus not related to the postulated phosphorylated intermediate of adenosine triphosphatase reaction.

REFERENCES


Note Added in Proof—Two recent papers by Gruda, Kakol and Rzymsko (Bull. Ac. Pol. Sci. 8, pp. 129 and 133, 1960) having a bearing on the subject matter of this communication have come to our attention. These authors describe studies on the binding of Pi to myosin, H-meromyosin, and reconstituted actomyosin, with the use of both P32- and P32-labeled ATP. They conclude that their data support the view that Pi bound to myosin and H-meromyosin is directly derived from ATP. However, inspection of their data with Pi32 clearly shows that Pi becomes bound, and the experiments with ATP32 are difficult to evaluate because of the correction that should be made for the binding of ATP to the protein (cf. ref. 5 of this paper).
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