Interaction of Actomyosin with Adenosine Triphosphate at Low Ionic Strength

II. FACTORS INFLUENCING CLEARING AND SUPERPRECIPITATION: ADENOSINE TRIPHOSPHATASE AND BIREFRINGENCE OF FLOW STUDIES*

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The clearing of actomyosin at \( \mu = 0.15 \) to 0.16 and pH 7.0 brought about by adenosine triphosphate in the presence of added magnesium was interpreted in the preceding paper in terms of the dissociation of actomyosin into its components (2).

This report deals with various factors influencing the clearing reaction and the accompanying changes in adenosine triphosphatase activity. Adenosine triphosphate was found to be most effective among the various nucleoside triphosphates and magnesium to be specific among the divalent metals tested. At \( \mu < 0.1 \), adenosine triphosphate and magnesium alone cannot produce clearing; chelating reagents or the relaxing factor of muscle are necessary. The possible physiological meaning of these findings is briefly discussed.

EXPERIMENTAL PROCEDURE

The preparation of myosin and actin was described previously (2).

The muscle granules were prepared as follows: rabbit muscle was homogenized in 3 volumes of 0.05 M KHCO₃ centrifuged for 20 minutes at 9,000 \( \times g \), and the supernatant was centrifuged for 1 hour at 35,000 \( \times g \). The precipitated granules were suspended in 0.05 M KHCO₃, clarified for 20 minutes at 9,000 \( \times g \), and used within 24 hours after the preparation.

The increase in the double refraction of flow, measured in an apparatus of the Edsall type, was used as a measure of clearing (2).

ATPase activity was determined in most experiments with the use of the pH-Stat system described in the preceding paper; in some experiments the \( P_i \) liberation was measured according to Fiske and SubbaRow (3).

C⁴-ADP-labeled F-actin was prepared according to Matsumori, Gouvea, and Gergely (4). Free ATP was removed by Dowex 1 treatment (5) instead of prolonged dialysis (4). Samples dried on filter paper strips were counted in a Packard Tri-Carb scintillation counter essentially as described by Löfffeld and Eigner (6).

Various nucleotides were purchased from Pabst Company and C⁴-ATP from Schwarz Laboratories. Polyethylene sulfonate was a kind gift of the Upjohn Company, New York. Heparin was purchased from General Biochemicals, Inc. Phosphoenolpyruvate and crystalline pyruvate kinase were obtained from Sigma Company. EGTA was purchased from Dojin Yakugaku Company, Kumamoto, Japan.

RESULTS

Substrate Specificity—The effect of various phosphate compounds on the clearing of actomyosin was tested in the presence of 0.15 M KCl and 1 mM MgCl₂. In 0.5 and 1 mM concentrations, only ATP was able to cause clearing. ITP, CTP, UTP and GTP gave rise to immediate superprecipitation, whereas ADP, AMP, IDP, IMP, PP, inorganic triphosphate, and hexapolyphosphate (Calgon) did not produce any change. The effect of increasing the concentration of various nucleoside triphosphates is shown in Fig. 1. ATP was most effective at all concentrations; CTP came next, and GTP was the least effective. At, or above, 5 mM the differences disappeared.

The effect of the ATP concentration on the clearing of actomyosin was studied in the presence of phosphoenolpyruvate:pyruvate kinase as an ATP-generating system. The results are shown in Fig. 2. Complete clearing was observed at 80 \( \mu \)M ATP. In contrast, ATP, without the generating system in concentrations less than 150 \( \mu \)M produces essentially immediate superprecipitation, and even at 150 \( \mu \)M superprecipitation is complete in 5 minutes. It is interesting to note that, in the absence of the generating system, 10 \( \mu \)M ATP produces only incomplete superprecipitation of actomyosin, but the same concentration of ATP, when maintained by the pyruvate kinase system, causes typical superprecipitation.

Effect of ADP and \( P_i \) on Clearing and Enzymatic Activity—To obtain a clearer view of the role of the ATP concentration in clearing and superprecipitation, and within the associated changes in the ATPase activity, we studied not only the dependence of the enzymatic rate on the initial ATP concentration, but calculated the concentration of ATP remaining at the time of superprecipitation. These results are shown in Fig. 3. It will be seen that the initial ATPase rate is essentially independent of the initial ATP concentration within the range of 0.5 to 2 mM. The

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The following abbreviations are used: EGTA, ethylene bis(N-diaethyl-\( \beta \)-aminoethyl) ether; PS, polyethylene sulfonate.
ATPase rate in the state of superprecipitation increases somewhat increasing initial ATP concentrations, and the rates do not greatly vary depending upon whether magnesium is present in equimolar amounts or in 1 mM excess. Somewhat surprisingly, the ATP concentration remaining in the system at the time of superprecipitation considerably increases with the initial ATP concentration. This suggested to us that there are factors controlling the onset of superprecipitation other than the lowering of the ATP concentration. To test this view, ADP and Pi, the reaction products of the ATPase reaction, were investigated. Fig. 4 clearly shows that addition of ADP greatly increased the initial rate of ATP hydrolysis and, concomitantly, superprecipitation was observed. Pi alone somewhat shortens the duration of the clear phase but has practically no effect on the initial phase; however, when it is added together with ADP the initial rate becomes quite rapid and superprecipitation takes place instantaneously. The ADP and phosphate effect cannot be due to the Mg++ available for complex formation with ATP since the addition of an excess amount of Mg++ with respect to ATP and phosphate did not change the results (see Fig. 4).

Metal Specificity—A limited survey was undertaken to determine whether the Mg requirement for clearing (2) is specific. Tested at 1 mM concentration, Mg alone was effective. One group of divalent metals did not interfere with rapid superprecipitation (Mn, Ca, Co, Ba, and Ni). Another group (Cd, Fe, Zn, Cu, and Hg) prevented superprecipitation, but, instead of clearing, the formation of a precipitate was observed. With 1 mM Mg present, further addition of 1 mM Ca or Ba did not prevent clearing; whereas Mn, Ni, and Co caused immediate superpre-
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-LOG [MgCl₂], M

FIG. 5. Effect of Mg concentration on the clearing of actomyosin. Conditions: 0.15 M KCl; 1 mM ATP; 0.01 M histidine; myosin, 0.24 mg per ml; and F-actin, 0.06 mg per ml. MgCl₂, as indicated on the abscissa. Total volume, 10 ml; pH 7.0; 20°. Key: ○, velocity gradient, 100 sec⁻¹; □, 50 sec⁻¹; Δ, 10 sec⁻¹.

FIG. 6. Effect of various metal ions on the ATPase activity. Conditions: 0.15 M KCl; 1 mM ATP; myosin, 1.0 mg per ml; and F-actin, 0.25 mg per ml. Metal concentration 1 mM. Total volume, 5 ml; pH 7.0; 20°. Ordinate: ATPase activity as total micromoles of Pi liberated, determined by alkali titration.

common initial point of both curves in Fig. 7 corresponds to the fact that in the absence of magnesium the rate is practically constant. As magnesium is added, the initial rate becomes slower and at the time of superprecipitation it exceeds that observed without magnesium. By varying the magnesium concentration between 0.5 and 5.0 mM, the initial rate remains at the same level, and the final rate at the same high, value. It would appear from these data that there is no obvious relation between the ATP and the magnesium concentrations since the maximal clearing effect can be obtained both with excess ATP and with excess magnesium provided the total magnesium exceeds 0.1 mM (cf. Fig. 5). An obvious corollary of these observations is the fact (also shown in Fig. 7) that, as the ATPase rate in the clear phase drops, the duration of the clear phase increases, presumably because of the persistence of higher ATP concentration during the entire period.

The effect of varying the KCl concentration is shown in a similar way in Fig. 8. With both the magnesium concentration and the initial ATP concentration being 1 mM in all the experiments at all KCl concentrations, a gradual decrease in the initial rate is observed with increasing KCl concentration, whereas the ATPase rate at the time of superprecipitation is essentially independent of the KCl concentration. Again, there is an inverse relationship between the duration of the clear phase and the initial ATPase rates.

Effect of -SH Reagents—It has been well known that -SH blocking reagents such as CMB, Salyrgan, and oxarsan inhibit superprecipitation (7). However, if CMB or Salyrgan were added to actomyosin before the addition of ATP, under such conditions that in the absence of mercurials clearing takes place, the subsequent addition of ATP leads to immediate superprecipitation (Fig. 9). When the mercurial is added after ATP, in the clear phase, only a small decrease in double defraction of flow is observed. As shown in Fig. 10, the effect of Salyrgan is reversed by β-mercaptoethanol. Addition of CMB or Salyrgan
Fig. 8. Effect of KCl concentration on the ATPase activity and superprecipitation of actomyosin. Conditions: 1 mM MgCl₂; 1 mM ATP; 1.2 mg of myosin per ml; and 0.3 mg of F-actin per ml. KCl concentration, as shown on the abscissa. Total volume, 5 ml; pH-Stat set for 7.0; 20°. Left ordinate: ATPase activity as micromoles of P_i liberated per minute per mg of myosin, determined by alkali titration. Right ordinate: time elapsed till onset of superprecipitation. Key: ○, maximal activity at the time of superprecipitation; ◦, initial activity during the clear phase; X, time before superprecipitation.

Fig. 9. Effect of CMB on the clearing of actomyosin. Conditions: 0.15 M KCl; 1 mM MgCl₂; 1 mM ATP; 0.01 M histidine; myosin, 0.46 mg per ml; and F-actin, 0.13 mg per ml. Total volume, 10 ml; pH 7.0; 20°. Velocity gradient, 50 sec⁻¹. Key: ◦, CMB was added before ATP addition; ○, CMB was added after ATP addition. Ordinate: double refraction of flow increase indicates clearing.

decreased somewhat the birefringence of the myosin aggregate; about 50% decrease was observed in the presence of 1 mM CMB or Salyrgan. On the other hand, the birefringence of F-actin was not affected by these reagents within 30 minutes of incubation.

Adding small amounts of Salyrgan to actomyosin from 0.1 to 2.6 moles per 10⁵ g of myosin, the duration of the clearing phase is gradually decreased and the ATPase activity increased. The increase is observable only in the superprecipitation stage which, with lower Salyrgan concentration, is preceded by a clear phase. A further increase of the Salyrgan to myosin ratio, although not affecting superprecipitation, depressed ATPase activity. When the Salyrgan to myosin ratio is 5 moles to 10⁴ g of myosin, superprecipitation is inhibited and ATPase activities are completely lost (Fig. 11).

Fig. 10. Reversal of the Salyrgan effect on the clearing of actomyosin by β-mercaptoethanol. Conditions: 0.15 M KCl; 1 mM MgCl₂; 1 mM ATP, 0.01 M histidine; myosin, 0.45 mg per ml; and F-actin, 0.15 mg per ml. Total volume, 10 ml; pH 7.0; 20°. Actomyosin was treated for 5 minutes with 5 moles of Salyrgan per 10⁵ g of myosin and various amounts of β-mercaptoethanol were added before ATP addition. In the control, Salyrgan treatment was omitted. Birefringence, shown on ordinate, was measured at a velocity gradient of 50 sec⁻¹. Key: ◦, Salyrgan treated; ○, control.

Fig. 11. Effect of Salyrgan on the ATPase activity of actomyosin. Conditions: 0.15 M KCl; 1 mM MgCl₂; 1 mM ATP; myosin, 1.0 mg per ml; and F-actin, 0.28 mg per ml. Total volume, 5 ml; pH-Stat set for pH 7.0; 20°. The numbers attached to the curves show moles of Salyrgan per 10⁵ g of myosin. Ordinate: ATPase activity as total micromoles of P_i liberated, determined by alkali titration. The arrow shows onset of superprecipitation.

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Clearing Brought about by Relaxing Agents—Ebashi² has first shown that the superprecipitation of natural actomyosin by ATP in 0.05 M KC1 and 1 mM MgCl₂ is delayed by EDTA and superprecipitation is preceded by clearing.

In view of the similarity between the effect of EDTA and other relaxing agents on myofibrils and glycerol-extracted muscle fibers (8, 9) and in view of Mueller’s studies (10) concerning the effect of muscle granules on the volume of actomyosin precipitates, we undertook some studies on the clearing of actomyosin by various relaxing agents.

S. Ebashi, personal communication.
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TABLE I

Effect of relaxing agents on clearing of reconstituted and natural actomyosin

<p>| Conditions: 4 mM MgCl₂, 1.6 mM ATP, 0.01 M histidine, and proteins as follows: reconstituted actomyosin (0.3 mg of myosin and 0.07 mg of F-actin per ml); natural actomyosin (0.3 mg per ml); crude muscle extract (0.66 mg per ml); supernatant of the extract (0.5 mg per ml), and muscle granules (0.18 mg per ml). When added, EDTA or EGTA concentration was 1 mM. The KCl concentration was 0.08 M and 0.05 M for the reconstituted and natural actomyosin, respectively. Total volume, 10 ml; pH 7.0; 20°. Double refraction of flow was measured at a velocity gradient of 50 sec⁻¹. |</p>
<table>
<thead>
<tr>
<th>Additions</th>
<th>Reconstituted actomyosin</th>
<th>Natural actomyosin</th>
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</thead>
<tbody>
<tr>
<td>Control</td>
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<tr>
<td>Muscle extract</td>
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<td>12</td>
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<tr>
<td>Supernatant</td>
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<td>13</td>
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<tr>
<td>EGTA</td>
<td>16</td>
<td>12</td>
</tr>
</tbody>
</table>

TABLE II

Effect of aging of F-actin on clearing and ATPase activity of myosin

<p>| Conditions: 0.08 M KCl; 4 mM MgCl₂; 2 mM ATP; 0.01 M histidine; myosin, 0.4 mg per ml; and F-actin, 0.1 mg per ml. When added, EDTA concentration was 1 mM. Total volume, 10 ml; pH 7.0; 22°. Fresh actin: F-actin 2 to 3 hours after polymerization. Aged actin: fresh actin was stored overnight at 2°C. Fresh myosin: myosin, 1 to 2 days after preparation. Aged myosin: myosin, 1 week after preparation. Birefringence was measured at a velocity gradient, 50 sec⁻¹. The ATPase activity is given as micromoles of Pi liberated per mg of myosin per minute, and was determined by the Fiske-SubbaRow method. |</p>
<table>
<thead>
<tr>
<th>System</th>
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<tr>
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<tr>
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<td>17</td>
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<tr>
<td>Fresh actin + aged myosin</td>
<td>15</td>
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<tr>
<td>Aged actin + fresh myosin</td>
<td>5*</td>
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<tr>
<td>Aged actin + aged myosin</td>
<td>3*</td>
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</table>

* Immediate superprecipitation was observed.

The data presented in Table I show that crude muscle extract, granules, EDTA, and EGTA, all known to have relaxing activity (cf. (8, 9)), inhibit superprecipitation and cause an increase in Δn characteristic of clearing. On the other hand, the supernatant obtained by clarifying a crude muscle extract at 35,000 × g for 1 hour and lacking relaxing activity (11), is also unable to produce clearing.

In the course of our work with EGTA we became aware of the fact that clearing could not always be obtained with reconstituted actomyosin, although superprecipitation never failed to take place. As shown in Table II, this seems to be related to some changes taking place in F-actin on aging. EGTA was found to be effective even when EDTA was not.

The specific clearing effect of a given substance can be observed only in a rather narrow range of KCl concentration. As illustrated in Fig. 12 for EGTA, it appears that at too low KCl concentration, e.g. 0.04 M, superprecipitation inevitably occurs; at the other end of the scale, KCl 0.1 M, ATP alone causes clearing.

The reversal of the action of relaxing agents by Ca++, well known for myofibrils and glycerol-extracted muscle fibers (cf. (7)) is also found in the case of clearing of actomyosin. Fig. 13 illustrates this point for EGTA. Similar results were obtained with granules and with EDTA.

Separation of F-actin from Actomyosin by Polyethylene Sulfonate and Heparin—In addition to high ATP, EDTA, and other chelating compounds, and muscle granules, Bárány and Jaiale (12) have found a group of charged compounds that cause inhibition of muscle fibers and of actomyosin in the presence of small amounts of ATP. PS is one of them; they afford another piece
of perhaps indirect evidence for the dissociation of actomyosin under conditions of relaxation.

Since PS forms a precipitate with myosin, clearing cannot be observed directly. But experiments with actin containing C\(^4\)-ADP as a marker show that although myosin precipitates, actin becomes soluble (Table III). This conclusion has been reached earlier by Bárány and Jaisle (12), somewhat indirectly, by studying the effect of the PS supernatant on the Mg-activated myosin-ATPase, and on the viscosity of myosin. However, it should be mentioned that neither PS nor heparin could release F-actin largely from the already superprecipitated actomyosin, as seen in Table III.

**DISCUSSION**

The clearing of actomyosin by ATP at low ionic strength has been interpreted in the preceding paper (2) in terms of dissociation into F-actin and myosin. As shown in the present study, ATP and Mg appear specific for this phenomenon at \(\mu = 0.16\).

It is interesting to note that Mg, Ni, and Co override the clearing effect of Mg and cause immediate superprecipitation. The fact that the effect of a given metal was the same, both when histidine was present and when it was not, suggests that complex formation with the imidazole group of histidine (13) did not interfere with the metal-protein, or metal-ATP, interaction. It is interesting to note that ATP was found to be the most effective nucleoside triphosphate for relaxation of muscle fibers in the presence of the muscle-relaxing factor (14). This special position of ATP in these rather different contexts may be fortuitous, since in the work described above the various nucleoside triphosphates react directly with actomyosin, whereas, in the case of the relaxing factor system, the specificity may be on the level of the production of the relaxing substance (15, 16) by granules.

Whether ATP produces clearing or immediate superprecipitation depends greatly on its concentration. Since ATP is constantly hydrolyzed by actomyosin, the study of this concentration dependence requires a generating system to obtain reliable measurements. The concentration of ATP sufficient for clearing of actomyosin (89 \(\mu\)M) in the presence of excess phosphoenolpyruvate and pyruvate kinase leads to rapid superprecipitation in the absence of the generating system. In the latter case, the concentration of ATP also determines the duration of the clearing phase, if added in high enough concentration to produce clearing. It appeared at first that the superprecipitation sets in when the concentration of ATP in the system at the time of superprecipitation varied inversely with the initial ATP concentration. This suggested that, if more ATP was initially present, the products of ATP hydrolysis may promote superprecipitation. This was found to be the case: addition of ADP and P\(_i\) to clear actomyosin produced rapid superprecipitation. These findings may represent the molecular counterpart of the observations of Lorand and Molnar (8) that ADP inhibits relaxation in the myofibrillar system, and may also explain why phosphoenolpyruvate and pyruvate kinase (as well as other rephosphorylating enzymes) promote clearing of actomyosin (see above) and relaxation of myofibrils or fiber bundles (8, 17, 18).

At a somewhat lower ionic strength, <0.1, ATP and Mg by themselves cannot produce clearing. However, addition of relaxing granules causes clearing, as well as the addition of EDTA (first shown by Ebashi) and other similar chelating agents. It is most likely that the clearing produced by these agents also depends on dissociation, as that produced by ATP at a somewhat higher ionic strength. It should, however, be pointed out that whereas clearing produced by relaxing agents is reversed by Ca\(^{++}\), that produced by ATP alone is not. The dissociating effect of other reagents (termed interaction inhibitors (12)), causing relaxation of glycerol-extracted muscle fibers and inhibition of Mg-activated actomyosin ATPase, has been previously demonstrated (12) and more directly confirmed in this paper for PS and heparin.

In all of these experiments there seems to be a good correlation between superprecipitation and high ATPase activity on the one hand, and clearing and low ATPase activity on the other. This would be consistent with the view that clearing is due to dissociation of actomyosin as discussed above and in the preceding paper.

The fact that EDTA neither inhibits the ATPase activity nor causes clearing of actomyosin, when aged F-actin is used, might be related to the previous finding of Perry and Grey (19), who failed to observe an EDTA effect in the reconstituted actomyosin. We have confirmed that EDTA is always effective with natural actomyosin (19). Recently Weber and Winicur (20) found that with some actin preparations, even EGTA does not work with reconstituted actomyosin. The reason remains to be elucidated.

The effect of mercurials described in this paper (with small amounts accelerating superprecipitation and stimulating ATPase, larger amounts inhibiting both superprecipitation and clearing as well as ATPase) is perhaps related to the activation of myosin-ATPase first described by Kielley and Bradley (21). Similar observations have been made recently by Tonomura,
Yoshimura, and Kitagawa (22), although they fail to relate these changes to the altered interaction between myosin and actin, but emphasize the loss of Ca normally bound to myosin. The enhancement of superprecipitation by mercurials with a concomitant loss of the bound Ca of myosin is somewhat at variance with views that assign a role to Ca in promoting superprecipitation and, by analogy, contraction (cf. (8, 20)).

The relation between superprecipitation and contraction has been repeatedly stressed in the literature (cf. (23)). To settle the question of the extent to which clearing is an analogue of relaxation, a view that is being increasingly expressed, and thus a suitable tool for the study of factors controlling it in vivo will require further experimentation.

**SUMMARY**

In the presence of 0.15 M KCl, adenosine triphosphate and Mg are specific for the clearing response of actomyosin which is accompanied by low adenosine triphosphatase activity. Blocking of a few sulfhydryl groups by appropriate reagents (0.5 to 10 moles per mole of myosin) accelerates the onset of superprecipitation; a similar acceleration is obtained by adding adenosine diphosphate. Superprecipitation is always accompanied by increased adenosine triphosphatase activity. The results have been discussed in terms of the dissociation and reformation of actomyosin.

**REFERENCES**


interaction of actomyosin with adenosine triphosphate at low ionic strength: ii. factors influencing clearing and superprecipitation: adenosine triphosphatase and birefringence of flow studies

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