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EFFECT OF MYOSIN ON ACTIN-BOUND NUCLEOTIDE EXCHANGE IN THE PRESENCE AND ABSENCE OF ATP*

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SUMMARY

We have continued our investigation of bound nucleotide exchange in F-actin and actomyosin by measuring the release of actin-bound [^{3}H]ADP using a rapid ultrafiltration technique. In agreement with previous work, the rate of actin-bound nucleotide release was found to be greatly increased by myosin in the presence of ATP under conditions of superprecipitation. However, we now find that the same effect of myosin is observed not only in the presence of ATP but also in the presence of ADP where no superprecipitation occurs. This effect of myosin is abolished at high ionic strength, and heavy meromyosin does not show the effect, suggesting that aggregation of the myosin is essential. We conclude that the increased exchangeability of the actin-bound nucleotide in actomyosin is unrelated to the ATPase activity or contraction of the actomyosin, but is caused simply by the binding of F-actin to myosin filaments.

INTRODUCTION

The polymerization–depolymerization process in actin has interested muscle biochemists for many years, but it still remains unclear whether this process plays any role in the contraction cycle in muscle. In the hope of detecting structural changes in the actin polymer associated with its interaction with myosin during superprecipitation, several laboratories have investigated the exchange of the actin-bound nucleotide in actomyosin[1–7]. Such studies have shown that the exchange of the actin-bound nucleotide with free ATP is indeed increased by myosin[3–7], suggesting that the actin might undergo a transient depolymerization as part of the contraction cycle.

It has been difficult, however, to establish a quantitative correlation between the increase in nucleotide exchange and the superprecipitation or ATPase of actomyosin or myofibrils. The increased exchange does not correlate in time with the turbidity change in superprecipitating actomyosin[5]; it often stops before reaching completion even when ATP hydrolysis by the actomyosin is continuing[3,5]; and the rate of nucleotide exchange does not change with the ATPase rate when the ATP concentration is varied[3]. Furthermore, it has been reported that appropriate modification of

* A preliminary report of some of this work has appeared previously[21].

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myosin can completely inhibit the actomyosin ATPase without diminishing the effect of myosin on the actin–nucleotide exchange[8]. These observations cast doubt on the idea that the nucleotide exchange or the F to G transformation of actin plays any direct part in the contraction cycle.

In further investigating this problem, we have now observed that the enhancement of F-actin–nucleotide exchange by myosin occurs even when the ATP in the medium is replaced by ADP so that no ATP hydrolysis or superprecipitation can occur. On the other hand, the effect of myosin does seem to depend on the aggregation of the myosin because it is abolished when the ionic strength is increased, and heavy meromyosin, which cannot form filaments, does not enhance the exchange of nucleotide in F-actin even at low ionic strength.

METHODS

Protein preparations

The customary procedures of this laboratory were used for the preparation of myosin, heavy meromyosin, actin, and creatine kinase (ATP: creatine phosphotransferase, EC 2.7.3.2)[9–12]. All protein concentrations were determined by ultraviolet spectrophotometry.

To introduce radioactive nucleotide into the actin, washed F-actin pellets were suspended at a final concentration of 5–8 mg/ml in 10 mM imidazole–HCl buffer (pH 7), containing 0.5 mM [^{3}H]ATP, and the solution was sonicated repeatedly in an ice-bath to bring about complete nucleotide exchange. Duplicate aliquots were withdrawn at this point for determination of the specific radioactivity of the total nucleotide. The free nucleotide was then removed by two treatments with 1% (w/v) Dowex 1-X4 anion-exchange resin, and the solution was brought to 1 mM in $MgCl_2$ and 50 mM in KCl to polymerize the actin. A second pair of aliquots was then taken for determination of the specific activity of the actin-bound nucleotide.

To determine the specific activity of the nucleotide in the samples referred to above, the protein was precipitated and the bound nucleotide released by $HClO_4$, the extracts were neutralized with NH_4OH, and the nucleotide concentration and radioactivity in the extract were measured by ultraviolet spectrophotometry and liquid-scintillation counting, respectively.

For all the actin preparations used in this work, the specific activity of the actin-bound nucleotide was equal, within 10%, to that of the total nucleotide in the actin solution just before Dowex treatment, showing that the actin-bound nucleotide was uniformly labeled. The final actin preparations contained 19–22 μmoles bound nucleotide per g of protein.

Liquid-scintillation counting

The counting solution used was a 1:1 (v/v) mixture of toluene and 2-ethoxyethanol, containing 6 g 2,5-diphenyloxazole, 0.25 g *p*-bis-[2-(5-phenyloxazolyl)]-benzene, and 20 g "Cab-o-sil" per l. Aqueous samples of 1 ml were dissolved in 15 ml of this solution, and 0.1 ml of 5 M NH_4OH was added to induce gelation of the "Cab-o-sil". The use of "Cab-o-sil" thixotropic gel was found to give more stable count rates, presumably by preventing a slow precipitation of nucleotide on the walls of the counting vials. The measured count rates were adjusted for slight variations

in quenching by recounting each sample after addition of a small volume of [^{3}H]ADP solution as an internal standard; but, in fact, the counting efficiency in this system was not much affected by the composition of the samples, even by the presence of the NH_4ClO_4 in the actin extracts, provided the volume of aqueous sample was always 1.0 ml.

Measurement of bound-nucleotide release

In the present work, we have investigated the exchangeability of the actin-bound nucleotide by measuring the rate of release of bound [^{3}H]ADP originally present in the F-actin. This approach has the advantage that measurements can be made directly by counting the radioactivity of protein-free ultrafiltrates, in contrast to the lengthy process of washing actomyosin sediments and extracting the bound nucleotide which is required when incorporation of free labeled nucleotide is measured[5,6]. The amount of [^{3}H]ADP released into solution after a given time was measured by sampling the protein-free ultrafiltrate of the reaction mixture by rapid filtration through an Amicon XM-50 "Diaflo" membrane. An Amicon Model 50 ultrafiltration chamber was used, which had been modified to reduce the filtrate volume beneath the membrane. The reaction mixture was incubated in a flask in a thermostat bath at 25°, and at an appropriate time after the addition of actin to the mixture, a 10-ml aliquot was transferred to the Amicon chamber, which was immersed in the same thermostat bath. Nitrogen at 50 lb/inch^2 was applied to the chamber, the first ml of filtrate was discarded to flush out the chamber and two successive samples of about 1.2 ml were collected. Exactly 1.0 ml of each of these filtrate samples was counted as described above. Collection of each sample took about 2 min, and the sample time indicated in the graphs is measured to the midpoint of this period. The remaining mixture in the Amicon chamber was discarded and the chamber dismantled and rinsed thoroughly before filtering the next aliquot of the reaction mixture.

To convert the count rate of each ultrafiltrate sample to the amount of free radioactive nucleotide in the reaction mixture at that time, it was necessary to correct for the fact that the XM-50 membrane does not pass nucleotide freely but consistently retains about 10–15% of the free nucleotide in the filtered volume. For each ultrafiltrate sample, the fraction of the free nucleotide actually transmitted by the membrane was determined by measuring the total nucleotide concentration in the filtrate spectrophotometrically before counting and dividing it by the known concentration of nucleotide added to the reaction mixture. The count rate of the sample was divided by this ratio to obtain the count rate of free nucleotide in the mixture. Division of this result in turn by the specific activity of the initial actin-bound nucleotide gave the molar concentration of released radioactive nucleotide, which was then expressed as a percentage of the total actin-bound nucleotide to give the "percent release" shown in the graphs which follow.

Although the XM-50 membrane has a nominal exclusion limit of 50000 molecular weight, which slightly exceeds the molecular weight of G-actin, we have found in control experiments that G-actin is completely retained. Also, the ultraviolet absorption spectra of the experimental ultrafiltrates showed no evidence of any protein, so we conclude that all the radioactivity of the ultrafiltrates did indeed represent free nucleotide.

RESULTS

Our first experiments were designed to determine whether ATP is actually required for the enhancement of actin–nucleotide exchange by myosin. The release of bound [^{3}H]ADP from actin and actomyosin was compared in the presence and absence of free ATP. The ATP in the latter case was replaced by an equal concentration of cold ADP to dilute the released [^{3}H]ADP so that measurements of nucleotide release would not be complicated by rebinding of released [^{3}H]ADP. In the experiment shown in Fig. 1, low ionic strength and low nucleotide concentration were used, in the presence of Mg^{2+}, to imitate the conditions of our previous studies of nucleotide incorporation[5]. For the solid curves, the free nucleotide was maintained as ATP by creatine kinase and phosphocreatine, while for the dashed curves, the creatine kinase was omitted so that the free nucleotide remained as ADP. As expected, the result in the presence of ATP corresponded to the earlier observations of nucleotide incorporation[3,5,6], showing that the technique used here does provide an equivalent measure of nucleotide exchangeability. Of great interest, however, is the finding in Fig. 1 that the effect of myosin was the same in the absence of creatine kinase as in its presence even though no superprecipitation was observed in the latter case. The mere binding of myosin, under conditions where no ATP was present and no ATP hydrolysis or superprecipitation occurred, appeared to produce the same acceleration of bound nucleotide release as was observed during superprecipitation.

There is a possibility in Fig. 1 that the exchange in the absence of added creatine kinase might have been due to the presence of a small amount of creatine kinase as a contaminant, which could produce ATP at a rate sufficient to cause ex-

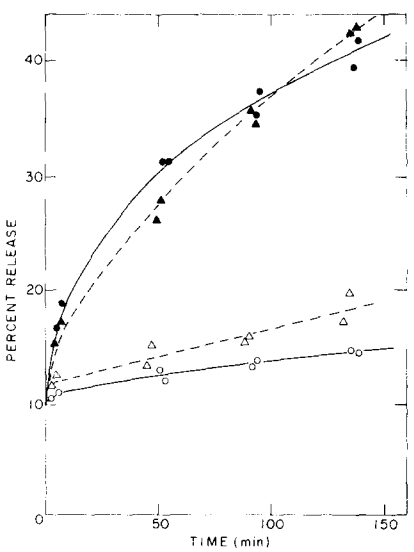

Fig. 1. Effect of myosin on the release of bound nucleotide from actin in the presence and absence of creatine kinase. All reaction mixtures finally contained 50 μM ADP (or ATP), 10 mM phosphocreatine, 4 mM $MgCl_2$, 28 mM KCl, 20 mM imidazole buffer (pH 7.0), and 0.2 mg/ml actin added at time zero. ●, ▲, 1.2 mg/ml myosin added immediately after actin; ○, △, no myosin added. ●, ○, 0.2 mg/ml creatine kinase added (free nucleotide converted to ATP); ▲, △, no creatine kinase added (free nucleotide remained as ADP).

change without causing noticeable superprecipitation. Therefore the experiment was repeated omitting phosphocreatine as well as creatine kinase from the samples in which the free nucleotide was ADP. Here again, as shown in Fig. 2, the effect of myosin was seen with ADP (dashed curves) was well as with ATP (solid curves); in fact, the release of bound nucleotide was greater in the former case, suggesting,

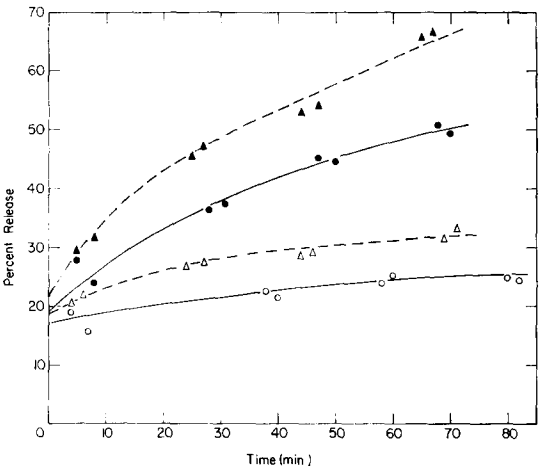

Fig. 2. Effect of myosin on the release of bound nucleotide from actin in the presence and absence of phosphocreatine. All reaction mixtures finally contained 50 μM ADP (or ATP), 2 mM $MgCl_2$, 20 mM imidazole buffer (pH 7.0), and 0.2 mg/ml actin added at time zero. ●, ▲, 1.0 mg/ml myosin added immediately after actin; ○, △, no myosin added. ●, ○, 0.2 mg/ml creatine kinase and 10 mM phosphocreatine added, KCl concn. = 38 mM; ▲, △, no creatine kinase or phosphocreatine added, KCl concn. = 68 mM.

perhaps, that phosphocreatine has a specific stabilizing effect on F-actin similar to the reported effect of inorganic phosphate[13]. Finally, to eliminate the possibility that the exchange with ADP resulted from the production of ATP by contaminant adenylate kinase (ATP:AMP phosphotransferase, EC 2.7.4.3), an experiment was carried out in the presence of a magnesium-chelating agent to remove any free Mg^{2+} and thereby block adenylate kinase activity[14] (Fig. 3). The bound nucleotide release from F-actin as well as actomyosin was faster in the absence of free divalent cations than in the previous experiments, but nevertheless, myosin was still seen to have a strong effect on the rate of nucleotide release. It seems clear from these experiments, then, that the effect of myosin on actin–nucleotide exchange is unrelated to the superprecipitation or ATPase activity of the actomyosin complex since it occurs equally well in the absence of ATP.

A possible explanation for the effect of myosin is that the binding of F-actin to myosin filaments produces a mechanical stress in the actin filament which increases the frequency of breaks in the polymer and hence accelerates nucleotide exchange, in a manner analogous to the effect of sonication[17,18]. In this case, the effect of myosin should depend on the presence of multiple actin-binding sites on each filament of aggregated myosin and should disappear when the ionic strength is increased enough to disaggregate the myosin. This prediction was tested, and it is clear in Fig. 4 that an increase in ionic strength does indeed reduce the ability of

myosin to accelerate the nucleotide release from F-actin, until the effect of myosin vanishes at an ionic strength of about 0.2. Actually, at high ionic strength, myosin may even have increased the stability of the F-actin somewhat. The change in ionic strength had no effect on the behavior of the F-actin alone. The effect of ionic strength on actomyosin was not due to dissociation of the actomyosin complex because parallel viscosity measurements (Table I), at an ionic strength higher than the maximum used in Fig. 4, showed a strong actin–myosin interaction in the presence of ADP.

A further test of the role of myosin filaments in the enhancement of actin–nucleotide exchange is provided by the use of heavy meromyosin in place of myosin, because heavy meromyosin does not form filaments at low ionic strength. An ex-

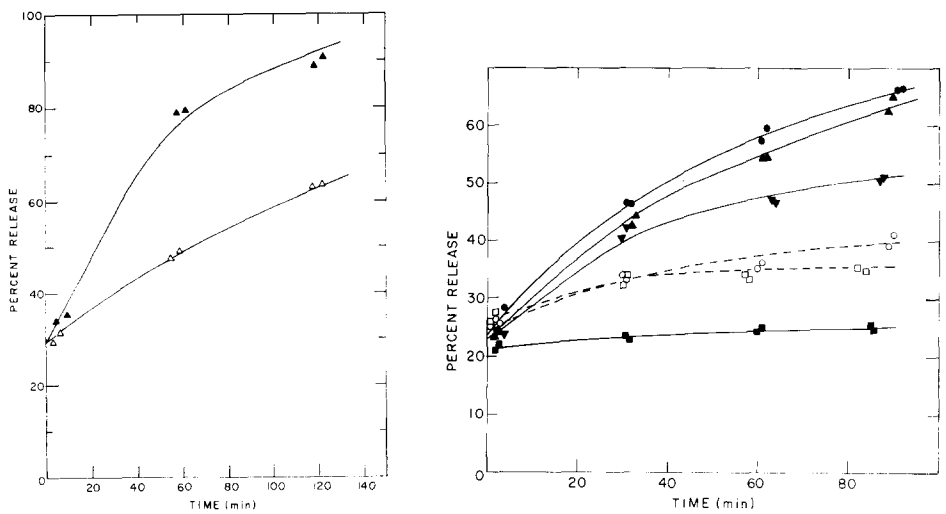

Fig. 3. Effect of myosin on the release of bound nucleotide from actin in the presence of the magnesium-chelating agent, 1,2-diaminocyclohexanetetraacetate. All reaction mixtures contained 100 μM ADP, 4 mM 1,2-diaminocyclohexanetetraacetate, 41 mM KCl, 10 mM potassium phosphate buffer (pH 7.0), and 0.3 mg/ml actin added at time zero. ▲, 2 mg/ml myosin added immediately after actin; △, no myosin added.

Fig. 4. Effect of ionic strength on bound nucleotide release from actomyosin and actin. All reaction mixtures finally contained 50 μM ADP, 4 mM $MgCl_2$, 10 mM imidazole buffer (pH 7.0), and 0.2 mg/ml actin added at time zero. Solid symbols, 1.2 mg/ml myosin added immediately after actin; open symbols, no myosin added. KCl concn. varied as follows: ●, ○, 0.05 M; ▲, 0.10 M; ▼, 0.13 M; ■, □, 0.22 M.

TABLE I

VISCOSITY TEST FOR ACTIN–MYOSIN INTERACTION AT HIGH IONIC STRENGTH

Conditions: 0.32 M KCl, 4 mM $MgCl_2$, 20 mM imidazole buffer (pH 7.0), 1.2 mg/ml myosin, 0.2 mg/ml actin, and, where indicated, 50 μM ATP or ADP. Viscosities were measured in a 5-ml Ostwald viscometer with an outflow time for water of 0.93 min at 25°. All measurements were averages of two samples. The first line gives the sum of values of log η_{rel} for actin and myosin measured separately.

	log η_{rel}		
	No nucleotide	*ADP*	*ATP*
Sum of actin *plus* myosin	0.154	0.158	0.156
Actin–myosin mixture	0.276	0.269	0.146

periment with heavy meromyosin in the presence of ATP is shown in Fig. 5, together with a simultaneous experiment with myosin under identical conditions. It is clear that, although the acto-heavy meromyosin mixture was hydrolyzing ATP at the same rate as the actomyosin, the heavy meromyosin had no effect whatever on the actin–nucleotide exchange rate. It has been reported that heavy meromyosin accelerates both polymerization and depolymerization in mixtures of F- and G-actin in the presence of ATP at low ionic strength[15], but evidently, if such an effect occurs under the strong polymerizing conditions used here (1 mM $MgCl_2$, 50 mM KCl), it does not produce a measurable increase in bound nucleotide exchangeability. The same result was also obtained with ADP in the medium in place of ATP (Fig. 6); if anything,

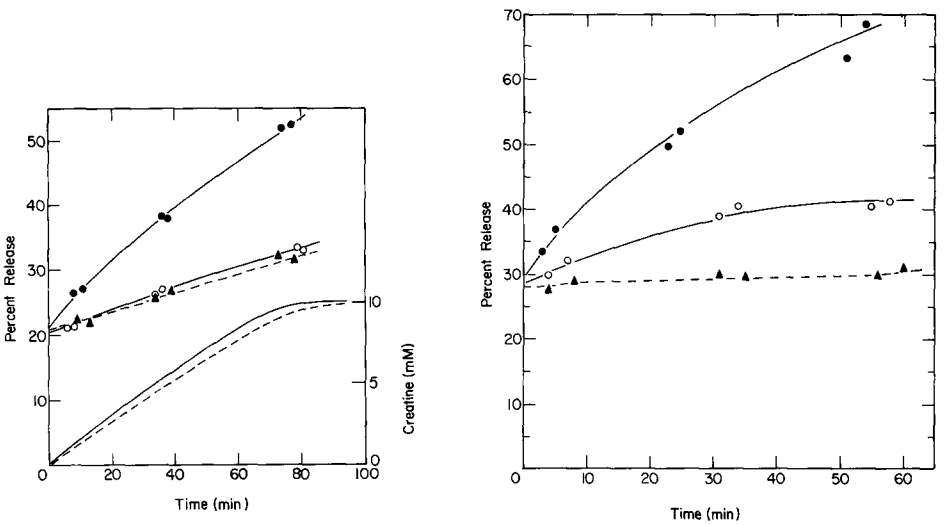

Fig. 5. Effect of heavy meromyosin on bound nucleotide release from actin in the presence of ATP. All reaction mixtures finally contained 50 μM ATP, 10 mM phosphocreatine, 0.1 mg/ml creatine kinase, 1 mM $MgCl_2$, 50 mM KCl, 20 mM imidazole buffer (pH 7.0), and 0.2 mg/ml actin. ○, actin alone; ●, 1 mg/ml myosin added at time zero; ▲, 1.15 mg/ml heavy meromyosin added at time zero. Lower curves: creatine liberation in the actomyosin (———) and acto-heavy meromyosin (------) samples, recorded by an automatic pH stat (ref. 12) simultaneously with the nucleotide release experiments.

Fig. 6. Effect of heavy meromyosin on bound nucleotide release from actin in the absence of free ATP. All reaction mixtures finally contained 50 μM ADP, 2 mM $MgCl_2$, 38 mM KCl, 20 mM imidazole buffer (pH 7.0), and 0.21 mg/ml actin added at time zero. ○, actin alone; ●, 1.0 mg/ml myosin added immediately after actin; ▲, 1.5 mg/ml heavy meromyosin added immediately after actin.

the heavy meromyosin may have decreased the actin–nucleotide exchange in this case, as was observed with myosin at high ionic strength in Fig. 4 (*cf.* ref. 16). It therefore seems clear that the accelerating effect of myosin on the exchange of F-actin-bound nucleotide, whether in the presence or absence of free ATP, results from the binding of myosin filaments to the actin.

DISCUSSION

Several laboratories have shown previously that the interaction of F-actin with myosin in superprecipitating actomyosin increases the rate of exchange of the

actin-bound nucleotide[3-7]. We have found here that myosin has this effect on F-actin not only when ATP is present but also when the free nucleotide is ADP so that no superprecipitation or ATP hydrolysis can occur. The effect seems to require only the binding of F-actin to myosin filaments.

While it has been generally assumed heretofore that the enhancement of actin–nucleotide exchange by myosin required superprecipitation[3,5,6], there is actually little experimental evidence with which our present findings conflict. The question has not been tested in any of the studies where the incorporation of free nucleotide into actomyosin or myofibrils was measured[3,5,6] because the free nucleotide in those experiments was always ATP. Our conclusion is also quite consistent with the finding that bound-nucleotide exchange in actomyosin is inhibited when superprecipitation is prevented by the tropomyosin–troponin system in the absence of Ca^{2+} (ref. 7) because under those conditions the actin and myosin would be dissociated. The only previous observations which seem contrary to our present findings are certain measurements of the release of bound radioactive nucleotide from reprecipitated actomyosin at high nucleotide concentration (3.3 mM)[3]. We have no ready explanation for the results of those experiments except to suggest that they may have been affected by the use of reprecipitated actomyosin instead of freshly mixed actin and myosin, by the relatively high nucleotide concentration used, or by differences in the protein preparations of our two laboratories. In the few attempts we have made, we have found it difficult to obtain reproducible results in such experiments.

In addition to our demonstration that the effect of myosin on actin-nucleotide exchange is independent of the presence of ATP, we have also shown that it requires that the myosin be aggregated. No increase in nucleotide exchange was seen with heavy meromyosin, or with myosin at high ionic strength. Therefore it seems likely that, as we have suggested before[5], the increased nucleotide exchange in the presence of myosin is due to increased mechanical stress on the actin filaments caused by their interaction with myosin filaments. In this sense, the effect of myosin may be like the effect of sonication[17,18]. Such mechanical stress might be caused simply by the participation of the actin filaments in an extensive, randomly cross-linked actomyosin gel which is continuously disturbed by the stirring of the suspension, or it may result more specifically from a regular side-by-side binding of actin filaments, with a helical half-pitch of 365 Å, to myosin filaments with a cross-bridge repeat of 429 Å (ref. 19).

With the observations reported here, we are left with no evidence that the actin-bound nucleotide or the G to F transformation of actin plays any role in the contractile cycle of muscle. Actomyosin ATPase and superprecipitation seem to be unaffected by substitution or even removal of the actin-bound nucleotide[6,20]; no transphosphorylation of the bound nucleotide has been detected[8,11]; and, as shown here, the exchangeability of the bound nucleotide seems unrelated to the superprecipitation or ATPase activity of actomyosin. Hence, the role of the actin-bound nucleotide in muscle may be solely to facilitate the polymerization of actin during myogenesis, or the repair of actin filaments which break in working muscle.

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