INTERACTION OF MYOSIN WITH MONOMERIC MATRIX-BOUND ACTIN

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Received 9 May 1973
Revised version received 25 May 1973

1. Introduction

Little is known about the interaction between monomeric actin (G-actin) with myosin; G-actin was said [1] not to activate myosin ATPase, but more recently [2] a small activation has been reported. When the ionic strength of an actin–myosin mixture is increased, the ATPase activity rises sharply as the actin polymerises. Possible reasons are (i) that the salt itself induces a conformation change in the actin, which increases both its ability to bind to myosin, and to associate to the F-form; (ii) that polymerisation leads to a new conformational state, i.e. traps the actin in a conformation, which interacts more efficiently with myosin, or (iii) that effective myosin-binding sites involve more than one actin monomer. A conformational difference between actin in the G- and F-states is suggested by a number of spectroscopic changes that accompany the polymerisation [3–6]. To prepare the putative monomeric actin in the F-conformation, we have coupled G-actin to a Sepharose matrix, so that when the ionic strength is increased any consequent conformational change cannot provoke polymerisation. We describe here now monomeric actin can be so coupled to a column without the loss of its functional characteristics, in particular its ability to bind its nucleotide ligand, and we show that the monomers are capable of binding myosin or subfragment-1 (S-1) in a specific manner, that the complex is dissociated by ATP, and that activation of myosin ATPase is small or absent.

2. Materials and methods

Rabbit skeletal muscle G-actin [7] was screened for purity by polyacrylamide gel electrophoresis in SDS [8]. Preparation of rabbit skeletal myosin and of S-1 followed the procedure of Lowey et al. [9].

The G-actin was coupled to Sepharose 4B after cyanogen bromide activation [10] at a low reagent concentration, so as to give a low coupling density [11]. For each column 25 ml of Sepharose suspension was used, which gives a bed volume of some 20 ml. To activate Sepharose for one column, 3.3 ml cyanogen bromide at 19 mg/ml is added to 25 ml of the suspension at room temperature, and the mixture is adjusted to pH 11 with 2 *N* NaOH, stirring for 6 min at 0°C. The gel is then washed with ice-water, followed by 450 ml of a buffer, containing 1.0 mM barbital, 0.25 mM ATP, 0.5 mM dithiothreitol and 0.2 mM $CaCl_2$, pH 8.6. 25 ml of G-actin at 1 mg/ml in this buffer is added and stirred for 16 hr in the cold. The gel is washed with 100 ml of the same buffer containing also 0.1 M *n*-hexylamine to eliminate any surviving active groups.

To estimate the protein bound to the matrix after washing, the most satisfactory method was found to be digestion with pronase, followed by colorimetric ninhydrin analysis, calibrated with G-actin. Aliquots of gel suspension (1 ml) with and without coupled actin, were treated with 1 ml pronase (Sigma Chemical Co.) at 10 μg/ml in 0.1 M borate, pH 5.5, and the mixture was incubated at 37°C for 2½ hr. The ninhydrin colour was developed by the standard method [12].

The nucleotide content of actin solutions was determined by dialysing into a buffer of 1.0 mM barbital, 0.5 mM ATP, 0.2 mM $CaCl_2$, pH 8.6, with no thiol

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reagent. The excess ATP in solution was then rapidly removed by passage through a short column of Dowex 1 in the chloride form. The concentration of G-actin was determined spectrophotometrically, using $E^{1\%}_{1\,cm} = 11.0$ [13]. An equal volume of perchloric acid was added and the concentration of nucleotide in the supernatant measured spectrophotometrically. (ϵ(P) = 10 000 at 259 nm [14]). The nucleotide content of the bound actin was measured in a similar way, except that labelled ATP was used. γ-^{32}P-ATP (Radiochemical Centre, Amersham) was incorporated into the G-actin in the last stage of its purification, 0.1 ml at a specific radioactivity of 3600 mCi/mmol being added per litre of G-actin after the final depolymerisation step. This concentration of label was maintained through all subsequent operations. Incorporation is rapid, in accordance with earlier findings [15]. After coupling, each gel column was washed at 4°C with 65 ml of a nucleotide-free buffer, containing 2.5 mM Tris, 0.2 mM $CaCl_2$, pH 8.2, to eliminate non-specific binding of ATP, and then eluted with 7% perchloric acid; 4.5 ml fractions were collected and nucleotide and total radioactivity were estimated from the absorbance at 259 nm and scintillation counting in a Packard Tri-Carb Counter.

Binding assays were conducted with S-1, its concentration being based on $E^{1\%}_{1\,cm} = 7.7$ at 280 nm [16]. An amount of protein roughly equivalent to the total of bound actin was applied to the column. The solvent was 2.5 mM Tris: 0.2 mM $CaCl_2$: 0.1 M KCl, pH 8.2. The gel was first freed of excess nucleotide and dithiothreitol by passing a sufficient volume of buffer to reduce the absorbance of the eluate to zero. The S-1 solution was then introduced and eluted with the above buffer. The quantity bound could be estimated from the difference between the amount applied and the amount eluted, but direct measurements were also made, by passing through the gel, in a jacketed column thermostated at 42°C, buffer containing 1% SDS to dissociate any complex. The protein emerging was estimated by spectrophotometry. Similar experiments were done with columns containing no S-1 to ensure that no actin or other ultraviolet-absorbing materials were eluted. Non-specific retention of S-1 was measured with a blank column containing no actin. Similar experiments were also performed with intact myosin.

To examine the effect of ATP on the actomyosin complex on the matrix, samples of gel and a control containing no actin were stirred with 25 ml of 2.5 mM Tris, 5 mM ATP, 5 mM $MgCl_2$, 0.1 M KCl, pH 7.0, at room temp. for 30 min. The gels were poured into columns and eluted with the Mg^{2+}—ATP-containing buffer. S-1 in each fraction was estimated as before by pronase digestion and ninhydrin analysis. ATPase measurements were made by quenching the reaction with perchloric acid to precipitate the protein, collecting the supernatant, and assaying for orthophosphate [17].

3. Results and discussion

The coupling procedure led to gels containing about 12 mg actin in a wet wt of 20 g of matrix. This was chosen to give a workable quantity of actin per column, while keeping the density of coupled protein molecules so low (one molecule of 10^5Å^3 volume per 10^8Å^3 of

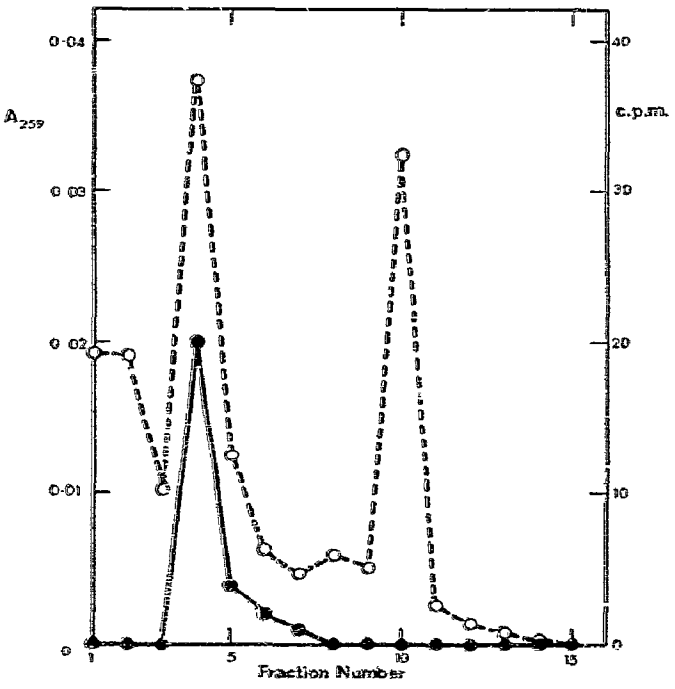

Fig. 1. Estimation of ATP retained by matrix-bound monomeric actin. After washing out excess nucleotide with nucleotide-free buffer, until the absorbance at 259 nm had fallen to zero, the column was eluted with perchloric acid. The eluted nucleotide in the fractions is estimated by ultraviolet absorbance (●—●—●), and by counting of the ^{32}P γ-label (o—o—o). The second peak of radioactivity is orthophosphate generated by hydrolysis of the ATP by the perchloric acid (see text).

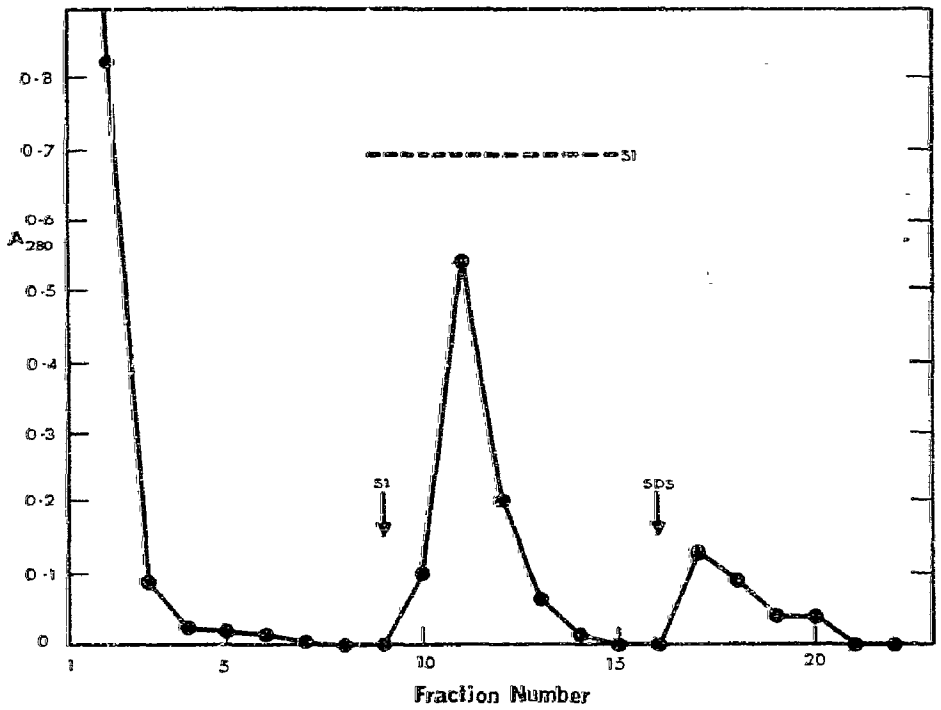

Fig. 2. Binding of myosin subfragment-1 to matrix-immobilised monomeric actin. The absorbance in the early fractions is due to excess nucleotide washed out of the column on changing to a nucleotide-free buffer. S-1 was introduced at the point indicated by the first arrow, and the unbound fraction was eluted in the first peak. Sodium dodecyl sulphate solution was then introduced in the column maintained at 42°C at the point marked by the second arrow, and the bound S-1 was eluted in the second peak. (o—o—o) Shows the concentration (absorbance) of the S-1 solution applied to the column.

column bed) as to render the possibility of interaction between actin monomers negligible. Analytical velocity sedimentattion, using schlieren optics, showed no detectable concentration of polymeric species in the G-actin preparations as used. That all the actin on the matrix was covalently bound was shown by the failure of aqueous buffers or the denaturant, SDS, to elute it from the column.

Since denaturation of actin leads to loss of the nucleotide [18], the retention of nucleotide was taken as an index of the native state. Results are shown in fig. 1. The excess nucleotide in the buffer and other absorbing materials having been driven out of the column, perchloric acid was passed through to denature the protein and elute the ATP. This procedure leads to some hydrolysis of ATP to orthophosphate. The absorbing peak has the spectrum of an adenine nucleotide, and coincides with a peak of radioactivity. The second radioactive peak is due to γ-phosphorus after hydrolysis. The separation of ATP (and ADP) from orthophosphate by the column, and the assignments of the peaks, were verified with a mixture of ATP and orthophosphate and a blank column containing no protein. That the orthophosphate indeed arises from hydrolysis by perchloric acid was also demonstrated by a control with ATP and a blank column. We find that up to 66% of the bound actin contains ATP, and is by this criterion native. (Moreover the nucleotide concentrations in fresh purified G-actin preparations were always markedly below 100%).

Binding of S-1 to the immobilised actin is shown in fig. 2. After passing through nucleotide-free buffer until the ATP absorbance falls to zero, S-1 was added, and the elution of the unbound fraction was followed spectrophotometrically. Binding was measured by difference and directly by elution with SDS at 42°C. This

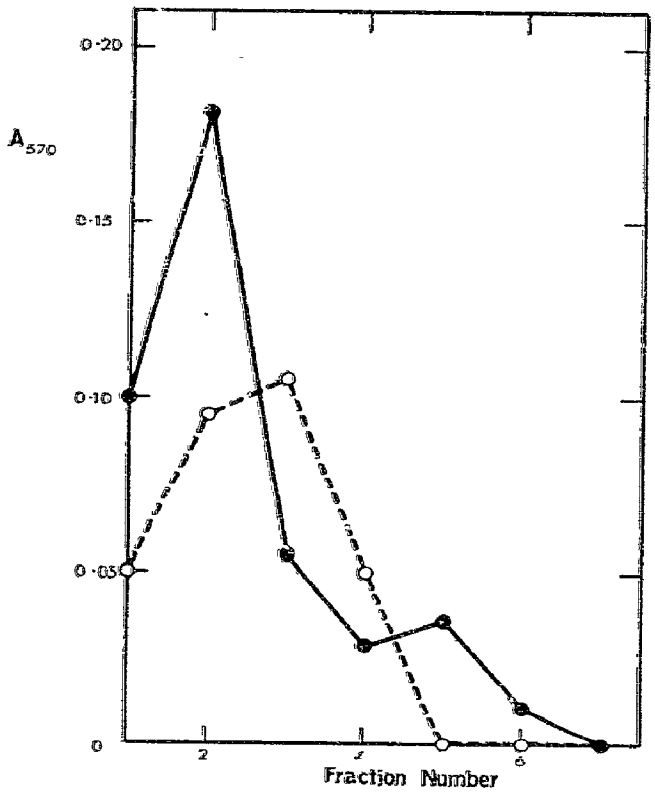

Fig. 3. Dissociation of the complex between matrix-bound monomeric actin and myosin S-1 by ATP. The absorbance refers to protein colour after digestion and addition of ninhydrin reagent. Equal volumes of myosin were applied to two columns, one containing bound actin (●—●—●), the other none (○—○—○). The eluate after addition of ATP was collected and analysed for protein (see text). The areas under the curves measure the protein solubilised by ATP.

is perhaps the most universal denaturant [18], which has no ill-effects on the column matrix, and has low ultraviolet absorption in the aromatic region. (6 M guanidine hydrochloride extracts an unknown ultraviolet-absorbing material from the Sepharose, which vitiated attempts at protein estimation). In general the mole ratio of S-1 retained to total bound actin was about 0.1. Relative to the active fraction this becomes 0.3 (corresponding to the typical result of fig. 2). The controls showed that not more than about 5% of the bound S-1 could be accounted for by non-specific adsorption. Thus the monomeric actin binds myosin heads with high affinity. Experiments with intact myosin led to qualitatively similar results, but the binding was substantially lower, largely no doubt because of steric obstruction.

Elution with a buffer containing magnesium and ATP leads to recovery of all the bound S-1 in the eluate. Alternatively identical volumes of S-1 were passed through columns of Sepharose with and without bound actin. The columns were allowed to drain, and then suspended in the ATP-containing buffer, after which the gels were repacked into the columns and eluted with more of the same buffer. Fractions were collected and analysed for protein. The areas under the curves (fig. 3) gave the amounts of S-1 on the columns, columns, and a consistent excess of the right order was found each time in the eluate from the actin-containing column. The pronase–ninhydrin analysis was necessary in all experiments involving ATP, the absorbance of which dominated the spectrum.

Attempts to determine whether ATPase was activated in this system were inconclusive, to the extent that any activation enhancement was clearly small; it seems likely in any case that the rate would be affected by diffusion effects in the gel, and modified rates of reassociation of the myosin, whose mobility will be greatly restricted in the matrix, with the bound actin. Attempts are being made to modify the coupling system to minimise these effects.

The activation of myosin ATPase by actin may depend on a conformational state of the latter, which occurs only in the F-form, or it may require the high local concentration of actin prevailing in the actomyosin complex, which would facilitate the repeated displacement of bound ADP from the myosin [19]. In order to establish the mechanism of the ATPase activation it is desirable to set up a model system which will allow characterisation of the intrinsic features of the actin–myosin interaction in monomeric species. The advantage of the approach which we have described is that it permits the use of physiological solvent conditions without the necessity of selective chemical inactivation of the actin–actin binding sites, of which there must be at least three kinds on each monomer, but not of the myosin attachment site. What our results so far have shown is that despite evidence that G-actin does not interact strongly with myosin [20], the isolated subunits, in conditions that would normally induce conversion to the F-state, do interact strongly with myosin heads: that, therefore, it is not the polymerisation as such which leads to activation

of the myosin attachment sites [20], and that interaction between isolated myosin and actin subunits is insufficient to provoke the large elevation of ATPase activity, which occurs in the condensed actomyosin phase.

Acknowledgements

We thank G.W. Offer and H. Baker for gifts of protein samples and helpful discussion. P.D.C. is supported by a Science Research Council Training Scholarship.

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