Biochem. J. (1975) **149**, 365–379
Printed in Great Britain

Affinity Chromatography of Immobilized Actin and Myosin

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(*Received* 12 *February* 1975)

Actin and myosin were immobilized by coupling them to agarose matrices. Both immobilized G-actin and immobilized myosin retain most of the properties of the proteins in free solution and are reliable over long periods of time. Sepharose–F-actin, under the conditions used in this study, has proved unstable and variable in its properties. Sepharose–G-actin columns were used to bind heavy meromyosin and myosin subfragment 1 specifically and reversibly. The interaction involved is sensitive to variation in ionic strength, such that myosin itself is not retained by the columns at the high salt concentration required for its complete solubilization. Myosin, rendered soluble at low ionic strength by polyalanylation, will interact successfully with the immobilized actin. The latter can distinguish between active and inactive fractions of the proteolytic and polyalanyl myosin derivatives, and was used in the preparation of these molecules. The complexes formed between the myosin derivatives and Sepharose–G-actin can be dissociated by low concentrations of ATP, ADP and pyrophosphate in both the presence and the absence of Mg^{2+}. The G-actin columns were used to evaluate the results of chemical modifications of myosin subfragments on their interactions with actin. F-Actin in free solution is bound specifically and reversibly to columns of insolubilized myosin. Thus, with elution by either ATP or pyrophosphate, actin has been purified in one step from extracts of acetone-dried muscle powder.

The potential of basing protein-separation methods on the molecular affinities found in biological systems has been greatly exploited in recent years [see review by Cuatrecasas (1972) and references cited therein]. Initially, attention was directed towards the development of affinity systems for the efficient purification of proteins but it was soon realized that affinity chromatography can be used to study binding interactions and the parameters affecting them. This led to more sophisticated approaches where affinity chromatography has been used to investigate enzyme mechanistic processes (Trayer & Olsen, 1972; Barker *et al.*, 1972; O'Carra & Barry, 1972), separation of isoenzymes (Brodelius & Mosbach, 1973) and as a means of probing the active sites of enzymes (Trayer & Trayer, 1974). Myosin, with its requirements to interact reversibly with actin, ATP and bivalent cations during muscle contraction, represents an ideal situation in which to use this methodology. The difficulties that can arise during a study of myosin–actin–ATP interactions, because of both the solubility properties of these proteins and their readiness to self-polymerize as well as to interact with one another, may possibly be minimized by immobilizing one or another of the reactants on to an agarose matrix. The resulting affinity adsorbents can then be used in a chromatographic procedure to investigate the parameters of these interactions.

In this study, we report on some of the properties of matrix-bound actin and myosin conjugates. Immobilization of these proteins was shown not to drastically alter their biological properties. Attempts were made to distinguish between Sepharose conjugates containing actin bound in its monomeric form, i.e. globular or G-actin, and the polymerized form found in myofibrils, i.e. the fibrous or F-actin. All the Sepharose–actin conjugates were found to react reversibly and specifically with myosin and its biologically active proteolytic-digestion products, heavy meromyosin and subfragment 1. The immobilized G-actin derivatives yielded the most satisfactory results and could be used to purify these fragments directly from proteolytic digests of myosin. These G-actin derivatives were particularly useful when studying the parameters affecting myosin–actin interactions and evaluating the results of chemical modification of these proteins. Some of this work has been briefly reported (Bottomley & Trayer, 1973).

The Sepharose–myosin conjugates were shown to reversibly bind F-actin and were used to isolate actin in a single step from extracts of acetone-dried muscle powder.

Materials and Methods

Materials

Chemicals. Sepharose-4B was purchased from Pharmacia (G.B.) Ltd., London W.5, U.K., and CNBr from R. Emanuel, Wembley, Middx., U.K. ATP was obtained from Kyowa Hakko Kogyo Co., Tokyo,

Japan, and all other nucleotides, *N*-ethylmaleimide and α-*N*-benzoyl-L-arginine ethyl ester hydrochloride were from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K. Iodoacetamide, unlabelled, was bought from Koch–Light Laboratories, Colnbrook, Bucks., U.K., and was recrystallized from light petroleum (b.p. 60–80°C) before use. Iodo-[1-^{14}C]acetamide was obtained from The Radiochemical Centre, Amersham, Bucks., U.K. All other chemicals were AnalaR grade and used as supplied except that organic solvents were dried over molecular sieves, type 4A (BDH Chemicals, Poole, Dorset, U.K.) for at least 48h before use.

Proteins. Papain (EC 3.4.22.2, from papaya latex) and bovine serum albumin were supplied by Sigma (London) Chemical Co.

Protein preparations. Actin was extracted and purified from the acetone-dried powder prepared from the minced back and leg muscles of New Zealand White rabbits (Bárány *et al.*, 1957) essentially as described by Spudich & Watt (1971). The purity of the actin preparations was monitored before use by electrophoresis on polyacrylamide gels in the presence of sodium dodecyl sulphate (Weber & Osborn, 1969).

Myosin was prepared from the longissimus dorsi muscles of New Zealand White rabbits by the method of Trayer & Perry (1966).

Heavy meromyosin was obtained from the parent myosin molecule by tryptic digestion (Szent-Gyorgyi, 1953) and further purified by gel filtration on Sephadex G-200 and by affinity chromatography on a Sepharose conjugate of 6-aminohexan-1-ol pyrophosphate (Trayer *et al.*, 1974*a*,*b*).

At the onset of this project, subfragment 1 was prepared from myosin by the cellulose–papain procedure of Lowey *et al.* (1969). Since this immobilized papain derivative proved to be unstable in our hands and was required to be freshly synthesized before every use, it was abandoned in favour of the more stable Sepharose–papain derivative (see below for preparation and assay). Similarly, in the early days of this project subfragment 1 was isolated by gel filtration on Sephadex G-200 from the digests (Lowey *et al.*, 1969). Subsequent work on affinity chromatography of muscle proteins from this laboratory (Trayer *et al.*, 1974*a*,*b*) has resulted in the subfragment 1 being isolated as a routine directly from the water-soluble fractions of a Sepharose–papain digest of myosin on the Sepharose–6-aminohexan-1-ol pyrophosphate derivative (Trayer *et al.*, 1974*a*). In a typical subfragment 1 preparation, Sepharose–papain (in an amount required to hydrolyse 0.4–0.8 μmol of α-*N*-benzoyl-L-arginine ethyl ester/min in the assay system described below) was added per ml of myosin (8–10mg/ml) dissolved in 0.5M-KCl–5mM-cysteine–2mM-EDTA, pH6.2. The resulting suspension was stirred for 15min at room temperature (usually 18–20°C) and the reaction stopped by cooling the reaction mixture and spinning down the Sepharose–papain in a low-speed centrifuge at 4°C. The supernatant was dialysed overnight against 5mM-triethanolamine–HCl, pH6.8, and any precipitated myosin etc. removed by centrifugation at 80000*g* for 30min. After adjusting the supernatant to pH7.5 with 0.1M-triethanolamine, the crude subfragment 1 was subjected to affinity chromatography on an agarose–6-aminohexan-1-ol pyrophosphate conjugate.

Tropomyosin and troponin-I were gifts from Dr. P. Cummins and Mr. H. Syska (respectively) of this department.

Methods

Enzyme assays. Measurements of the ATPase (adenosine triphosphatase) activities of myosin and its proteolytic subfragments were carried out either by a pH-stat method (Moos & Eisenberg, 1967) or by a direct determination of P_i (Fiske & SubbaRow, 1925) after incubation as described by Trayer & Perry (1966). Exact incubation conditions for these assays are given where appropriate in the text.

The activity of the Sepharose–papain was measured in a pH-stat (Radiometer Autotitrator TTT1c, Radiometer, Copenhagen, Denmark) by its ability to hydrolyse a synthetic substrate, α-*N*-benzoyl-L-arginine ethyl ester. A portion (0.1ml) of the enzyme was added to 1.9ml of 2.5mM-α-*N*-benzoyl-L-arginine ethyl ester containing 5mM-cysteine and 2mM-EDTA, pH6.2. The solution was maintained at pH6.2 by the titrant 20mM-NaOH (freshly prepared with previously boiled glass-distilled water).

Preparation of Sepharose–protein conjugates. The proteins were coupled to CNBr-activated Sepharose-4B essentially as described by Axen *et al.* (1967). All proteins were coupled to Sepharose activated by 100mg of CNBr/g of wet packed gel. Myosin was coupled to this activated gel overnight at 4°C in 0.5M-KCl containing 5mM-triethanolamine–HCl, pH8.5. After being washed, the gel–protein was suspended in 1M-ethanolamine–HCl, pH8.0, for 2h at room temperature. The substituted gel was washed extensively under suction in the coupling buffer (3 litres) and then with 0.1M-KCl containing 5mM-triethanolamine–HCl, pH7.5 (2 litres); it was stored in this buffer. Myosin was also coupled to Sepharose 2B and 6B but since these conjugates did not offer any apparent advantages over the Sepharose 4B matrix, the latter was used as a routine.

Papain was coupled to CNBr-activated Sepharose-4B in the presence of 0.1M-Na_2CO_3–$NaHCO_3$ buffer, pH9.5, overnight at 4°C. The coupled gel was washed under suction with this buffer and then suspended in ethanolamine as above. The Sepharose–papain was then washed in turn with 1M-KCl (2 litres) and 0.5M-KCl containing 5mM-cysteine and 2mM-EDTA, pH6.2 (1 litre). The immobilized papain was stored in

this latter buffer. After hydrolysis of the myosin in a subfragment 1 preparation, the Sepharose–papain was washed and stored as above before reuse.

G-Actin (2–4mg/g of gel) was coupled to the activated gel in the presence of 5mM-triethanolamine–HCl, pH8.5. For the coupling of F-actin this buffer included 50mM-KCl containing 2.5mM-$MgCl_2$. The substituted gels were then washed under suction alternately with 2M-KCl and either 5mM-triethanolamine–HCl, pH7.5 (Sepharose–G-actin), or 50mM-KCl–5mM-triethanolamine–HCl (pH7.5)–2.5mM-$MgCl_2$ (Sepharose–F-actin). The gels were stored in the appropriate second buffer.

The extent of the coupling procedure was checked as a routine by E_{280} measurements on the Sepharose-free filtrates before and after coupling. In the case of the Sepharose–papain no further estimations on the washed gel were made since the amounts of the immobilized enzyme required for subfragment 1 preparations were determined by enzymic assay (see above). For Sepharose–myosin and actin matrices, the efficiency of the coupling process was determined directly on the exhaustively washed gels by subjecting portions to amino acid analysis after hydrolysis for 24h in 6M-HCl at 105°C. In all cases the coupling efficiencies were over 70% and in a routine procedure 1–2mg of actin and 4–5mg of myosin were coupled per g of wet packed gel.

All substituted gels were stored in their appropriate buffers containing 0.1% sodium azide as a bacteriostatic agent.

Chromatographic procedures. All the affinity columns were operated at room temperature (usually between 18° and 20°C) except where specifically stated otherwise and at a flow rate of about 20ml/h. Unless indicated to the contrary, quantitative recoveries of the enzymes from the columns were always obtained.

Between runs, the Sepharose–actin and Sepharose–myosin columns were washed with at least 3–5 bed volumes of 1M-KCl followed by the appropriate starting buffer.

The progress of the adenosine nucleotide gradients used in this study was monitored spectrophotometrically on a Unicam SP.1800 u.v. recording spectrophotometer (Pye–Unicam, Cambridge, U.K.) by using an $\varepsilon_{259}^{\text{litre}\cdot\text{mol}^{-1}\cdot\text{cm}^{-1}}$ for AMP, ADP and ATP at pH7.0 of 15400 (Bock *et al.*, 1956).

Detection of protein eluted under a nucleotide gradient was determined by light-scattering measurements after precipitation of the protein. In the initial part of this study the light scattered at 360nm after mixing 1ml of column eluate with 0.5ml of 15% (w/v) trichloroacetic acid was taken as an estimate of protein concentration. A linear relationship between the E_{280} of subfragment 1 and/or heavy meromyosin preparations and the turbidimetric analysis was established. Myosin gave more variable results with this method and subsequently all protein determinations were made by the turbidimetric tannin micromethod (Mejbaum-Katzenellenbogen & Dobryszycka, 1959) where the protein was precipitated with tannic acid and the turbidity stabilized by gum arabic. This method gave reproducible results with all the proteins used in this study and was much more sensitive than the trichloroacetic acid precipitation procedure. Light-scattering was measured at 500nm in this procedure.

Chemical modifications of myosin. N-Carboxy-DL-alanine anhydride (used for polyalanylation of myosin) was prepared by suspending *N*-benzyloxycarbonyl-DL-alanine (26.2g, 0.117mol) in ice-cold anhydrous ether (1 litre) and adding PCl_5 (30g, 0.144mol). The resulting mixture was shaken, with cooling in an ice bath, for 30min when most of the solid had dissolved. This solution was quickly concentrated *in vacuo* at 40–50°C with the careful exclusion of moisture. The concentrate was then dried twice from anhydrous ethyl acetate, cooled in an ice bath, and light petroleum (b.p. 60–80°C), cooled to −12°C, added to precipitate the product. After storage at −12°C overnight the white crystals were collected, washed with cold light petroleum and dried *in vacuo* over anhydrous calcium sulphate. The final product (m.p. 42–44°C with decomposition) was obtained in 75–80% yield and was used without further characterization.

Polyalanylation of myosin was carried out essentially as described by Edelman *et al.* (1968) but with minor modifications. To 10ml of myosin (7mg/ml) in 0.6M-KCl containing 4mM-ATP and 4mM-$MgCl_2$, pH7.0, was added 9ml of 0.1M-potassium phosphate buffer, pH7.0, and 0.1ml of 2-mercaptoethanol. *N*-Carboxy-DL-alanine anhydride (150mg) dissolved in 1ml of dioxan was added to this, and the resulting solution was stirred at 4°C for 2h. The reaction mixture was then dialysed for 24h against 2 litres of 0.3M-KCl containing 5mM-triethanolamine–HCl, pH7.5, and then against 2 litres of 5mM-triethanolamine–HCl, pH6.8, overnight. Any precipitate was removed by centrifugation at 80000*g* for 30min and the supernatant used in the experiments outlined below.

Blocking the cysteine residues of myosin with iodo[1-^{14}C]acetamide was carried out as follows. To myosin (5ml, 10mg/ml) dissolved in 0.5M-KCl–25mM-triethanolamine–HCl, pH7.9, was added 0.2ml of 10mM-iodo[1-^{14}C]acetamide (approx. 0.45 μCi/μmol). After mixing and leaving at 4°C for 30min, the reaction was quenched by the addition of 4vol. of 0.5M-KCl–5mM-2-mercaptoethanol–10mM-triethanolamine–HCl, pH7.9, and the myosin was immediately precipitated by adding to 20vol. of water at 4°C. The precipitated myosin was collected by centrifugation and dissolved in 0.5M-KCl–5mM-cysteine–2mM-EDTA, pH6.2, and subfragment 1 prepared from it as described above but with longer

dialysis times to ensure removal of any remaining excess of iodoacetamide. Heavy meromyosin and subfragment 1 were treated with between 5- and 10-fold molar excesses of iodo[1-^{14}C]acetamide in 25mM-triethanolamine–HCl, pH8.0, at 4°C. The reaction was quenched by the addition of an equal volume of 5mM-2-mercaptoethanol dissolved in this buffer followed by a short dialysis (3–4h) against 10mM-triethanolamine–HCl, pH7.8, containing 3mM-2-mercaptoethanol and then dialysis overnight against 5mM-triethanolamine–HCl (pH7.8)–0.1mM-2-mercaptoethanol. The extent of labelling was controlled by adjusting the time of reaction before quenching. Usually this was between 30 and 60min.

Inactivation of heavy meromyosin by *N*-ethylmaleimide was carried out at 4°C in a reaction solution containing heavy meromyosin (2ml, 6mg/ml) and *N*-ethylmaleimide (1ml, 10mM) dissolved in 25mM-triethanolamine–HCl, pH7.8. After 30min the reaction was quenched by the addition of 2-mercaptoethanol, and excess of reagents was removed by dialysis as described above.

Analytical procedures. Polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate was carried out, and gels were stained by the method of Weber & Osborn (1969). The stained gels were scanned on a Gilford recording spectrophotometer model 2000 fitted with a Gilford gel-scanning attachment, model 2410.

The sedimentation data were collected on a Beckman Spinco model E ultracentrifuge equipped with schlieren optics and by the use of a cell with a single-sector synthetic boundary centrepiece. Viscosity measurements were made in an Ostwald viscometer.

Radioactivity was measured in a Phillips liquid-scintillation analyser, model PW 4510, after mixing the samples with the scintillation fluid described by Brew *et al.* (1968).

Results

In contrast with actin in free solution, immobilization stabilized the molecule so that the columns could be used repeatedly over a period of 2–3 months before noticeable loss in their effectiveness occurred. Even so, attachment of actin to Sepharose does not appear to have as great a stabilizing effect on this protein as it does on many other proteins that retain their biological activities for considerable lengths of time. For example, if carefully stored in the presence of bacteriostatic agents, both the Sepharose–papain, used in this study to prepare subfragment 1, and the Sepharose–myosin have been kept without any appreciable loss of activity for over 6 months.

Biological activity of Sepharose–G-actin conjugates

Before proceeding with a study of the interactions of matrix-bound actin with myosin it was first decided to investigate the effect of the immobilization process on the integrity of the actin molecule. The immobilized G-actin still conferred a Mg^{2+}-stimulated ATPase activity to myosin, heavy meromyosin and subfragment 1 which was sensitive to increasing concentrations of KCl. This is shown in Fig. 1 where the relative ATPase activities obtained with either myosin (Fig. 1*a*) or subfragment 1 (Fig. 1*b*) and free F-actin are indistinguishable from those obtained with these preparations and the Sepharose-bound G-actin conjugate. The activation of the Mg^{2+}-stimulated ATPase activity of subfragment 1 and

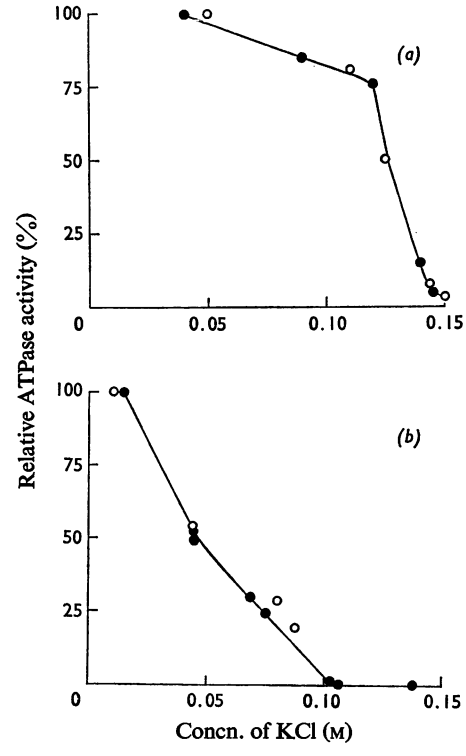

Fig. 1. *Variation with ionic strength of the Mg^{2+}-stimulated ATPase activity of complexes formed between free and Sepharose–actin with myosin and subfragment* 1

The assays were performed on a pH-stat at 25°C and pH7.5 in a final volume of 2ml containing 2.5mM-ATP and 5mM-$MgCl_2$. (*a*) Myosin (0.6mg/ml) was mixed with either free F-actin (0.4mg/ml) (●) or Sepharose–G-actin (equivalent to 3–3.5mg of actin) (○). (*b*) Subfragment 1 (0.3mg/ml) was mixed with the same amounts of free F-actin (●) or Sepharose–G-actin (○). Under these conditions the 100% values for the specific ATPase activities of these complexes (in μmol of P_i/min per mg of myosin component) were as follows: actomyosin, 0.85; Sepharose–actin–myosin, 0.1; actin–subfragment 1, 0.7; Sepharose–actin–subfragment 1, 0.15.

Table 1. *ATPase activity of heavy meromyosin–Sepharose–G-actin complexes in the presence of tropomyosin (TM) and troponin-I (TN–I)*

The assays were performed in a pH-stat at 25°C and pH 7.5 in a final volume of 2ml containing a final concentration of 2.5mM-ATP and 5mM-$MgCl_2$. Heavy meromyosin (0.5mg/ml) was incubated with Sepharose–G-actin (equivalent to 3–3.5mg of actin) in all experiments and either the tropomyosin and/or troponin-I added as indicated below. Under these conditions, the Mg^{2+}-stimulated ATPase activity of the Sepharose–G-actin–heavy meromyosin was about 0.1 μmol of P_i/min per mg of heavy meromyosin.

Added protein	ATPase activity of Sepharose–G-actin–heavy meromyosin complex (%)
No additions	100
TM (0.4mg)	100
TN–I (0.4mg)	70
TM (0.4mg)+TN–I (0.4mg)	45

myosin by the immobilized G-actin, although not as great as by the soluble F-actin (see legend to Fig. 1), still represented a considerable stimulation. The actin species bound to Sepharose in these experiments was judged to be G-actin from viscosity and analytical ultracentrifuge measurements before coupling to CNBr-activated Sepharose. Although the conditions used for coupling (5mM-triethanolamine–HCl, pH 8.6, for 16h at 4°C) would not be expected to promote polymerization, it cannot be entirely ruled out. Subsequent experiments in which F-actin was coupled to Sepharose (see the Materials and Methods section) also resulted in a polymer that activated the Mg^{2+}-stimulated ATPase activity of myosin and subfragment 1. The degree of activation found, however, was far more variable than that obtained with the Sepharose–G-actin derivative. In addition, when Sepharose–F-actin conjugates were used in chromatographic procedures to determine their capacity to bind subfragment 1 (see below) widely different values were obtained depending on the preparation used and its age. The Sepharose–G-actin conjugates always gave satisfactorily reproducible results in these experiments and were therefore used in the rest of the studies outlined in this paper.

Passage of more actin down columns of Sepharose–G-actin in the presence of 50mM-KCl and 2mM-$MgCl_2$ caused additional actin to bind to these columns suggesting that the immobilized G-actin was capable of polymerizing with free actin. Further washing of these columns with low-ionic-strength buffers, especially in the presence of ATP, effected a very gradual release of bound actin from the column. Not under any of these conditions did non-specific proteins such as bovine serum albumin show any tendency to be retained on the Sepharose–G-actin columns and appeared in the void volume.

Tropomyosin was retained on these immobilized G-actin columns but since the amount bound varied from one preparation of Sepharose–G-actin to another, no attempt was made to quantify this interaction. The interaction occurring between the tropomyosin and immobilized G-actin can be seen to be specific, however, from the results expressed in Table 1. These results show that the Mg^{2+}-stimulated ATPase activity of the Sepharose–actin–heavy meromyosin complex is partially inhibited by the inhibitory protein of the troponin complex (TN–I) and that this inhibition is enhanced in the presence of tropomyosin (Wilkinson *et al.*, 1972).

All of these results indicate that immobilization of actin has not grossly affected the ability of the immobilized molecule to polymerize with free actin or to interact with other myofibrillar proteins in a precise manner.

Binding of subfragment 1 to Sepharose–G-actin

Initially, experiments were conducted with the Sepharose–G-actin conjugate in a chromatographic procedure to determine the most suitable conditions for its interaction with subfragment 1. This 'single-headed' myosin fragment was used to simplify the interpretation of data. Binding of subfragment 1 was apparently enhanced when the columns were operated at room temperature rather than at 4°C. Inclusion of Mg^{2+} (2mM) in the application buffer (5mM-triethanolamine–HCl, pH 7.5) also improved binding, whereas Ca^{2+} (up to 5mM) had no apparent effect. Under these conditions there was no interaction between the Sepharose–G-actin conjugate and non-specific proteins such as bovine serum albumin and ovalbumin. The apparent capacity of these columns to bind subfragment 1 was determined by the frontal analysis technique operated under optimum conditions, i.e. at room temperature in the presence of Mg^{2+}. In this experiment (Fig. 2) a constant concentration of subfragment 1 was applied to the column until the concentration of the enzyme in the eluate approached that of the applied solution. In a similar but separate experiment, bovine serum albumin was passed down the column to determine the elution volume of material that does not interact with the immobilized G-actin. The difference in elution volume between the two experiments represents the the volume of subfragment 1 (of known concentration) interacting with a known amount of immobilized G-actin. Assuming the interaction occurs at a 1:1 molar ratio, then a value of between 15 and 25% of the actin monomers attached to the Sepharose was calculated as representing the amount of matrix-bound actin capable of interacting with subfragment 1. Although this value depends on the association

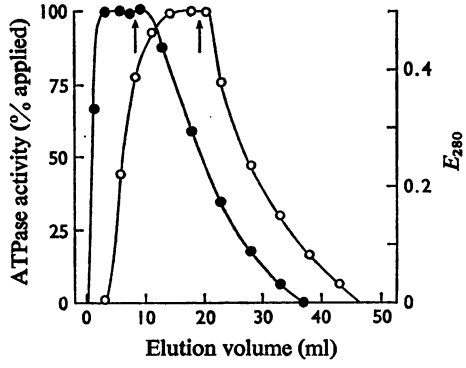

Fig. 2. *Frontal analysis chromatography of subfragment 1 on Sepharose–G-actin*

The column (8cm×1.2cm) was equilibrated with 5mM-triethanolamine–HCl, pH7.5, containing 2mM-$MgCl_2$ at room temperature. Bovine serum albumin ($E_{280} = 0.5$; ●) and subfragment 1 (1.1mg/ml; ○) were applied to the column in this buffer in separate experiments. The fractions were monitored for E_{280} (●) for the bovine serum albumin and for ATPase activity (○) for the detection of the subfragment 1. Samples (0.1ml) were assayed at 25°C for 5min with 5mM-$CaCl_2$, 2.5mM-ATP in 25mM-Tris–HCl, pH7.6, in a final volume of 2ml. P_i liberated was determined by the method of Fiske & SubbaRow (1925). At the arrows, the proteins were omitted from the buffers.

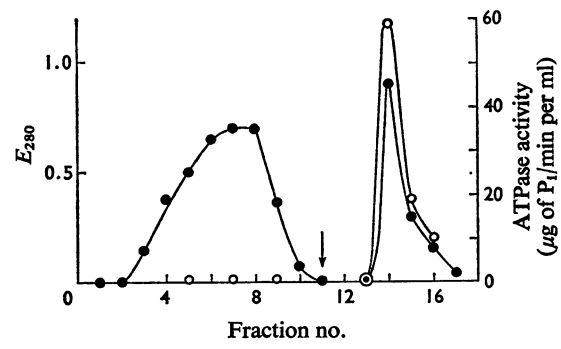

Fig. 3. *Chromatography of a Sepharose–papain digest of myosin on Sepharose–G-actin*

The water-soluble fraction (10ml, $E_{280} = 0.70$) of a Sepharose–papain digest of myosin was equilibrated with 5mM-triethanolamine–HCl, pH7.5, containing 2mM-$MgCl_2$ and applied to the column (10cm×1.2cm) equilibrated in the same buffer at room temperature. The column was operated at a flow rate of 20ml/h and 2.0ml fractions were collected. The arrow indicates where 0.25M-KCl was included in the developing buffer. ●, E_{280}; ○, ATPase activity (determined as in the legend to Fig. 2 except that column fractions were first dialysed to 5mM-triethanolamine–HCl, pH7.6, before assay).

constant occurring between the subfragment 1 and the actin in this system, it does provide a useful yardstick from which to work. This result was consistently found with several different preparations of Sepharose–G-actin.

The immobilized G-actin was capable of distinguishing between that fraction of protein in a subfragment 1 preparation that possessed enzymic (ATPase) activity and that which did not. In fact, these columns can be used to prepare active subfragment 1 direct from the water-soluble fraction of a Sepharose–papain digest of myosin (Fig. 3). The eluted protein migrated as a single peak in the analytical ultracentrifuge ($s^0_{20,w} = 5.75$) and its specific ATPase activity, in the presence of Ca^{2+} at pH7.6, was always higher (approx. 2.5 μmol of P_i/min per mg) than those preparations made by us using the conventional procedures of Lowey *et al.* (1969). In fact, subfragment 1 prepared by gel filtration from papain digests of myosin could be readily resolved into active and inactive (with respect to ATPase activity) fractions by passage down a Sepharose–G-actin column; the protein eluted from the column possessed a higher specific ATPase activity than that applied. The profile in Fig. 3 was obtained with only a small amount of starting material. This procedure has been scaled up for processing larger quantities of material but as a routine in our laboratory (R. C. Bottomley, H. R. Trayer & I. P. Trayer, unpublished work) subfragment 1 is prepared on a large scale by affinity chromatography on a Sepharose conjugate of 6-aminohexan-1-ol pyrophosphate (Trayer *et al.*, 1974*a*).

Dissociation of the immobilized actin–subfragment 1 complex could be achieved in a variety of ways (Fig. 4). Low concentrations of pyrophosphate (2–4mM), in the presence of equimolar amounts of Mg^{2+}, readily eluted subfragment 1 from these columns (Fig. 4*a*), this elution being much more efficient at 4°C than at room temperature. When the columns were operated at room temperature, low concentrations (2mM) of pyrophosphate alone, i.e. in the absence of Mg^{2+}, would not elute the bound subfragment 1. At 4°C, however, elution of the enzyme could be achieved by this concentration of pyrophosphate alone and this anion in the presence of Mg^{2+} (cf. Straub, 1943). Fig. 4(*b*) shows the dissociation of the immobilized complex when the column was irrigated with $Mg \cdot ATP^{2-}$. Both of these observations confirm the true specific nature of the interaction occurring between the matrix-bound actin and the subfragment 1. Raising the ionic strength by including KCl concentrations of greater than 0.15M in the developing buffer was also an effective way of eluting the bound protein (Fig. 4*c*). Inclusion of this concentration of KCl in the application buffer caused some

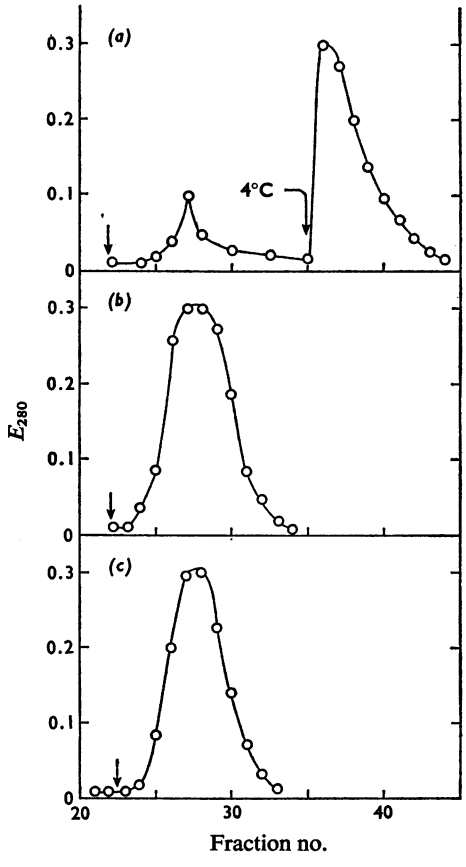

Fig. 4. *Elution of subfragment* 1 *from Sepharose–G-actin*

Subfragment 1 (3–3.5mg) was loaded on to the Sepharose–G-actin columns (8cm×1.2cm) equilibrated with 5mM-triethanolamine–HCl, pH7.5, containing 2mM-$MgCl_2$ at room temperature. After the columns had been washed with this buffer, the elution profiles obtained after irrigation of the columns with various additions to the basic buffer are shown. All columns were operated at a flow rate of 15ml/h and 2ml fractions were collected. The eluted fractions all displayed ATPase activity. ○, E_{280}. (*a*) '4°C' indicates where the column was stopped, placed in the cold for 1h, and then elution continued. Arrow denotes addition of 2mM-$Mg{\cdot}PP_i^{2-}$. (*b*) Arrow denotes the addition of 5mM-$Mg{\cdot}ATP^{2-}$. (*c*) Arrow denotes the addition of 0.5M-KCl.

80% decrease in the capacity of the column to interact with subfragment 1, the bound protein being readily eluted by further raising the KCl concentration [cf. conditions used by Chantler & Gratzer (1973)]. These observations are consistent with those shown in Fig. 1, in which the Mg^{2+}-stimulated ATPase activity of actomyosin-type complexes are inhibited by increasing KCl concentrations. In fact, if subfragment 1 is eluted from Sepharose–G-actin by a KCl gradient, then the concentration of KCl at which the peak amount was eluted was approximately the same as that shown in Fig. 1 for partial inhibition of the Mg^{2+}-stimulated ATPase activity of the actin–subfragment 1 complex. The most obvious explanation in the light of the column work is that KCl promotes dissociation of these protein complexes, thus inhibiting the Mg^{2+}-stimulated ATPase activity. [The specific Mg^{2+}-stimulated ATPase activity of myosin alone is very low (see Table 2) and would be negligible under the conditions described in the legend to Fig. 1.] In view of this, the later experiments were conducted in triethanolamine buffers adjusted to the required pH with acetic acid.

Parameters affecting the binding of heavy meromyosin and subfragment 1 *to Sepharose–G-actin*

This section of results deals with a more careful appraisal of the parameters affecting the interaction of subfragment 1 and heavy meromyosin with the immobilized G-actin.

Although the actin-binding and ATPase sites on myosin are closely related enzymically (e.g. the actin stimulation of the Mg^{2+}-stimulated ATPase activity of myosin), it is feasible that the binding sites for these molecules occur at different and distinct areas on the myosin head region and that during the preparation of subfragment 1 some species may be produced that only retain one of these properties. This hypothesis could be readily tested by subjecting the water-soluble fraction of a papain digest of myosin to affinity chromatography on Sepharose–G-actin (to test for actin binding) and on Sepharose–6-aminohexan-1-ol pyrophosphate (to test for pyrophosphate/ATP binding). Material binding to both columns was assumed to possess both properties and a search was made to find if any protein would bind reversibly to one of these columns but not to the other. In all experiments, however, protein which bound to one column bound quantitatively to the other and any material which passed unretarded through one column was also unable to bind to the other. Since these experiments strongly support the suggestion that the actin-binding and pyrophosphate (ATP)-binding sites on subfragment 1 are intimately related, no further attempt was made to differentiate between them by additional proteolytic-digestion studies.

The abilities of AMP, ADP and ATP to elute subfragment 1 from a Sepharose–G-actin conjugate were next compared. Stepwise elution by 10mM-ADP or -ATP (in the absence or presence of Mg^{2+}) was quantitative. On the other hand, 10mM-AMP^{2-} or -$Mg{\cdot}$AMP caused only very slight dissociation of the immobilized actin–subfragment 1 complex, and adenosine (up to 10mM) had no effect at all. A more careful comparison of the effects of ADP and ATP was made by gradient elution techniques. In the

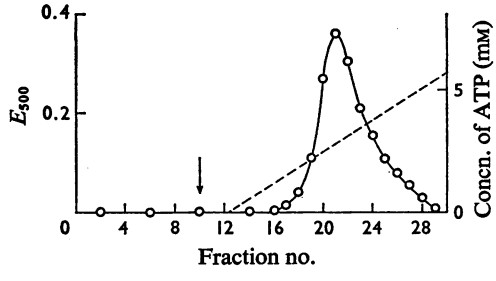

Fig. 5. *Elution by an ATP gradient in the presence of Mg^{2+} of subfragment 1 from Sepharose–G-actin*

The column (6cm×0.8cm) was equilibrated with 5mM-triethanolamine–acetate (pH7.5)–1mM-magnesium acetate at room temperature, and subfragment 1 (1mg), dissolved in this buffer, was applied. After being washed with this buffer, the column was developed with a linear gradient (at arrow) formed by 20ml of 5mM-triethanolamine–acetate (pH7.5)–1mM-magnesium acetate and 20ml of 10mM-ATP–12mM-magnesium acetate dissolved in 5mM-triethanolamine–acetate, pH7.5. A flow rate of 20ml/h was maintained, and 2ml fractions were collected. The fractions were monitored for protein (1ml portions were taken for the tannin turbidimetric micromethod), and the progress of the gradient was determined as described in the Materials and Methods section. ○, Subfragment 1; ----, ATP gradient concentration.

experiments reported in Fig. 5 and Table 2, the column size and dimensions, the amount of bound actin and the progress of the gradients were all kept constant. Fig. 5 shows a typical gradient elution of subfragment 1 from Sepharose–actin by $Mg{\cdot}ATP^{2-}$. The enzyme is eluted as a single peak although it does tend to trail. Sharper elution profiles were usually obtained when the bivalent cation was omitted from the gradient profile. Similar experiments were conducted by using other displacing ions, and the concentrations of the nucleotides required to elute the subfragment 1 are compared directly in Table 2. It should be pointed out that where no bivalent cation is given the disodium salt of ATP was used directly for the gradients. The inorganic cation-free ATP was prepared by passing the disodium ATP solution over Dowex-50 (H^+) (20–50 mesh) and adjusting to pH7.5 with 0.1M-triethanolamine. The elution profiles show, as expected, that ATP was better at dissociating the actin–subfragment 1 complex than ADP, which was as good as pyrophosphate. It was surprising, however, that those nucleotides were more effective elutants in the absence of Mg^{2+} than in its presence. This was not due to their acting as chelating agents and binding the Mg^{2+} used in the application buffers since EDTA (10mM) did not cause any elution of the bound subfragment 1.

Table 2. *Concentrations of adenosine nucleotides required to elute subfragment 1 from Sepharose–G-actin under various conditions*

The columns were operated exactly as described in the legend to Fig. 5. In all cases the samples were applied in 5mM-triethanolamine–acetate, pH7.5, containing 1mM-magnesium acetate, to a Sepharose–G-actin column (6cm×0.8cm) equilibrated in that buffer. After washing the column with this buffer, elution by gradients containing Mg^{2+} was carried out directly. For those gradients where Mg^{2+} was omitted, the columns were first subjected to a Mg^{2+}-free buffer wash (10ml) before application of the gradients. All columns were operated at room temperature.

Eluent	Concentration of displacing ion required for elution (mM)
ATP	1.80
ATP (inorganic cation-free)*	1.00
Mg·ATP	2.75
ADP	3.20
Mg·ADP	4.00
PP_i	3.20

* ATP was first treated with Dowex 50 (H^+ form); in all other cases the disodium salt of ATP was used.

From chemical modification studies, it is well established that there are two particularly reactive cysteine residues per subunit of myosin, generally referred to as the $-SH_1$ and $-SH_2$ groups, which are located at or near the active site of this enzyme (Sekine *et al.*, 1962; Sekine & Kielley, 1964). Blocking of the $-SH_1$ thiol group results in an activation of the Ca^{2+}-stimulated ATPase activity (Sekine & Kielley, 1964) whereas blocking both the $-SH_1$ and $-SH_2$ thiol groups eliminates all ATPase activities (Yamaguchi & Sekine, 1966). Thus the Ca^{2+}-stimulated ATPase activity of myosin, after reaction with a 25-fold molar excess of iodo[1-^{14}C]acetamide for 30min at 4°C (see the Materials and Methods section), was found to have increased two- to three-fold, suggesting that the $-SH_1$ cysteine groups had been labelled. When subfragment 1 was prepared from this material by digestion with Sepharose–papain, measurements of the radioactivity indicated that 1.08mol of iodoacetamide had been incorporated/mol of enzyme, confirming this observation. This labelled subfragment 1 readily bound to the Sepharose–G-actin conjugate and could be eluted by ATP etc. Its behaviour on this immobilized G-actin was virtually indistinguishable from that of the unlabelled enzyme. The SH_1-blocked fragment also bound to the Sepharose–6-aminohexan-1-ol pyrophosphate derivative confirming the accessibility of the substrate (pyrophosphate)-binding site.

If heavy meromyosin was treated with iodo[1-^{14}C]-acetamide in the same manner as myosin (see the

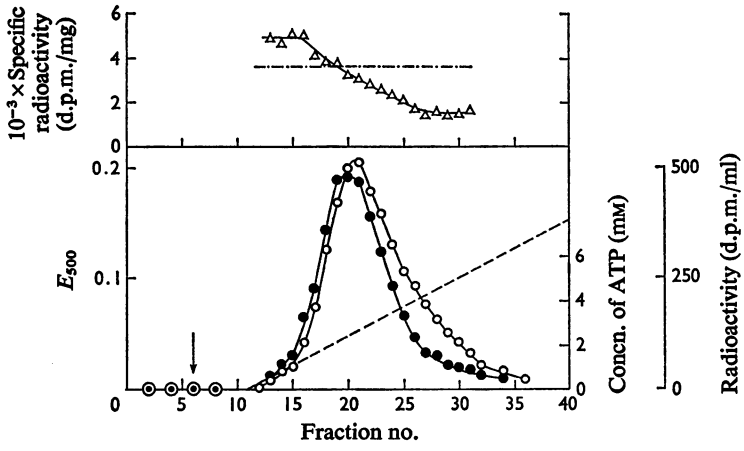

Fig. 6. *Chromatography of heavy meromyosin labelled with iodo*[1-^{14}C]*acetamide and unlabelled heavy meromyosin on Sepharose–G-actin*

Heavy meromyosin was treated with iodo[1-^{14}C]acetamide (see the Materials and Methods section) until 1.57mol of radioactive label was incorporated/mol of protein. Some 2.4mg of this material was mixed with 0.6mg of unlabelled protein and the whole, in 3ml of 5mM-triethanolamine–acetate, pH7.5, containing 1mM-magnesium acetate, was applied to a Sepharose–G-actin column (8cm×0.6cm) equilibrated with the same buffer. After the column had been washed with this buffer a linear gradient, formed from 20ml of the triethanolamine buffer and 20ml of 7.5mM-ATP and 8.0mM-magnesium acetate dissolved in that buffer, was applied (at the arrow); the column was operated at a flow rate of 20ml/h and 1.4ml fractions were collected. Samples (0.2ml) were taken for protein determination by the tannin turbidimetric microassay (○) and 1ml samples for radioactive counting (●). The progress of the gradient (---) was determined as described in the Materials and Methods section. The specific radioactivity (△) across the peak is also shown; -·-·, specific radioactivity of the applied protein.

Materials and Methods section), then, although radioactive label was incorporated into this fragment, no activation of the Ca^{2+}-stimulated ATPase activity was noticed. In fact, no effect on the Ca^{2+}-stimulated ATPase activity was noticed when up to 1.57mol of iodoacetamide was incorporated/mol of protein. In this case, however, a 40% decrease of the NH_4^+-stimulated (EDTA) ATPase activity was noted. Similarly, direct labelling of subfragment 1 with this reagent also caused a slight decrease in the Ca^{2+}-stimulated ATPase without any apparent activation when as little as 0.2mol of iodoacetamide was incorporated/mol of protein. The reason for these differences in labelling the native molecule and its fragments probably lies in the nature of the fragments themselves and their method of production. Nevertheless all of these labelled fragments were capable of interaction with both the immobilized G-actin and pyrophosphate derivatives. Iodoacetamide-labelled heavy meromyosin did show a slightly decreased ability to bind to the Sepharose–G-actin, which suggested a slight decrease in the association constant of this modified molecule for actin (Fig. 6). In this experiment, heavy meromyosin (1.57mol of iodo[1-^{14}C]acetamide incorporated/mol of protein) was mixed with unlabelled enzyme and applied to Sepharose–G-actin. Gradient elution of this material showed a slight displacement of the radioactive and protein peaks with a concomitant decrease in the specific radioactivity across the peak.

N-Ethylmaleimide is the reagent that has probably been most widely used to study the thiol groups of myosin [see, e.g., Reisler *et al.* (1974) and references therein]. All of the ATPase activities of heavy meromyosin were completely inactivated by the treatment of this protein with a large excess of this reagent. This inactivated fragment, however, still bound relatively strongly and reversibly to both the immobilized G-actin and pyrophosphate derivatives. These observations are consistent with the -SH_1 and -SH_2 cysteine residues of myosin not being located directly in either the actin-binding or ATP-binding sites.

Binding of myosin to Sepharose–G-actin

Myosin dissolved in 0.5M-KCl–5mM-triethanolamine–HCl buffer, pH7.6, passed unretarded through our immobilized G-actin columns equilibrated with this solvent. If the concentration of KCl in these buffers was decreased to 0.15M, a concentration at which myosin is sparingly soluble in dilute solution, then myosin would bind to these columns. The bound

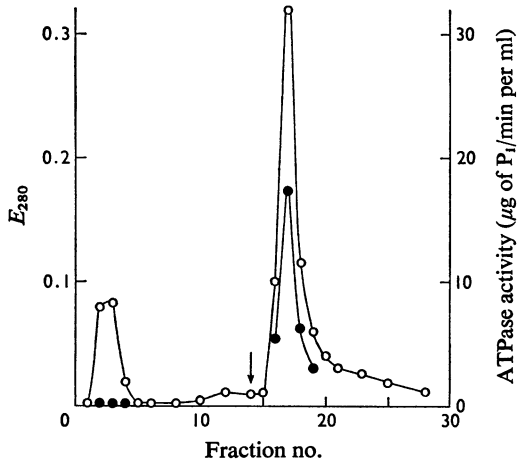

Fig. 7. *Chromatography of polyalanyl myosin on Sepharose–G-actin*

Polyalanyl myosin (2ml, $E_{280} = 0.95$), dissolved in 5mM-triethanolamine–HCl, pH7.5, containing 2.5mM-$MgCl_2$, was applied to the column (10cm×0.8cm) equilibrated with the same buffer at room temperature. The column was operated at a flow rate of 20ml/h and 2ml fractions were collected. The arrow indicates where 0.5M-KCl was included in the developing buffer. The same result was obtained when either 5mM-ATP or -pyrophosphate (in the presence of Mg^{2+}) was used as the eluent. ○, E_{280}; ●, ATPase activity (determined as in the legend to Fig. 2).

myosin could be eluted by low concentrations (2–4mM) of pyrophosphate and ATP, in the presence of Mg^{2+}, as well as by raising the ionic strength of the developing buffer. Although these observations suggest that a specific interaction has occurred between the myosin and the immobilized G-actin, a non-specific precipitation of the myosin on the column at this low ionic strength followed by resolubilization by the polyanions cannot be ruled out.

If the myosin is first rendered soluble at low ionic strength by treatment with *N*-carboxyalanine anhydride (Edelman *et al.*, 1968) then its specific interaction with the Sepharose–G-actin could be readily demonstrated. When the polyalanyl myosin was applied to a G-actin column at low ionic strength (Fig. 7), protein possessing no ATPase activity passed unretarded through the column whereas the biologically active modified myosin was tightly bound to the column and was only released when some specific displacing solute was introduced into the developing buffer. Since the polyalanylation procedure caused some 20–30% decrease in the specific ATPase activity of the myosin, it was noteworthy that the material retained on the column possessed the same specific ATPase activity as the enzyme before modification. This clearly demonstrates that the polyalanylation

Table 3. *ATPase activities of Sepharose–myosin*

The assays were performed on a pH-stat at 25°C and pH7.5. In the final assay mixture (2ml) were contained 1.25mM-ATP, 0.05M-KCl and either myosin (0.25mg) or the Sepharose–myosin (equivalent to 1mg of myosin) together with the various activating cations listed below. The Sepharose–myosin contained 5mg of myosin/g of wet packed gel.

Activating conditions	Specific ATPase activity (μmol of P_i/min per mg of myosin component)	
	Sepharose–myosin	Myosin
Ca^{2+} (2.5mM)	0.25	1.30
Mg^{2+} (2.5mM)	0.02	0.01–0.03
Mg^{2+} (2.5mM)+F-actin (0.5mg)	0.04	0.8–1.0

procedure causes irreversible denaturation of some of the myosin and not an overall decrease in the molecules' ability to hydrolyse ATP. It is also noteworthy that the introduction of numerous polyalanine peptides on to the surface of the myosin molecule does not noticeably affect its ability to interact with actin. Thus myosin, albeit modified, will interact specifically with the immobilized G-actin.

Previous work from this laboratory (Trayer *et al.*, 1974*a*,*b*) has shown that myosin dissolved in KCl would not bind to columns packed with immobilized ADP derivatives (Trayer *et al.*, 1974*a*) but would bind if the Cl^- anions in the solvent were replaced by acetate. When myosin, dissolved in either 0.6M-potassium acetate or ammonium acetate, was applied to a Sepharose–G-actin column, however, the myosin still passed through unretarded. Similarly, neither heavy meromyosin nor subfragment 1, dissolved in the high-ionic-strength acetate buffers, would interact with the matrix-bound actin. In fact, if these biologically active fragments of the myosin molecule were bound to Sepharose–G-actin at low ionic strength (conditions given in legend to Fig. 3) then they were readily eluted by including either potassium acetate or ammonium acetate (0.25M) in the developing buffer. Thus the interaction between myosin (and the myosin-like fragments) and the Sepharose–G-actin is very sensitive to the concentration of salt in the developing buffers.

Interactions of matrix-bound myosin

Myosin can also be immobilized to a CNBr-activated Sepharose matrix and shown to retain its biological activities. Table 3 describes the ATPase activities of this Sepharose–myosin when compared with free myosin under various conditions. The specific Ca^{2+}-stimulated ATPase activity of Sepharose–myosin was generally about 20% of that expected on

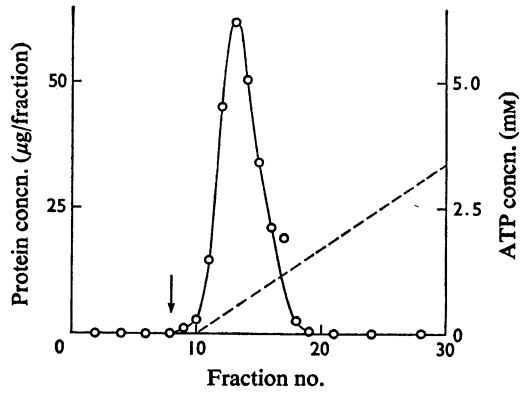

Fig. 8. *Chromatography of F-actin on Sepharose–myosin*

F-Actin (0.5ml, 2.5mg) in 50mM-KCl containing 10mM-triethanolamine–HCl, pH7.5, and 2.5mM-$MgCl_2$ was applied to a column of Sepharose–myosin (7cm×0.9cm) equilibrated in that buffer. The column was operated at 4°C at a flow rate of 15ml/h and 1.5ml fractions were collected. The arrow indicates where a linear gradient, formed from 25ml of the above buffer and 25ml of 5mM-ATP dissolved in this buffer, was applied to the column. The protein concentrations (○) were determined by the tannin turbidimetric microprocedure (see the Materials and Methods section); ----, ATP gradient concentrations.

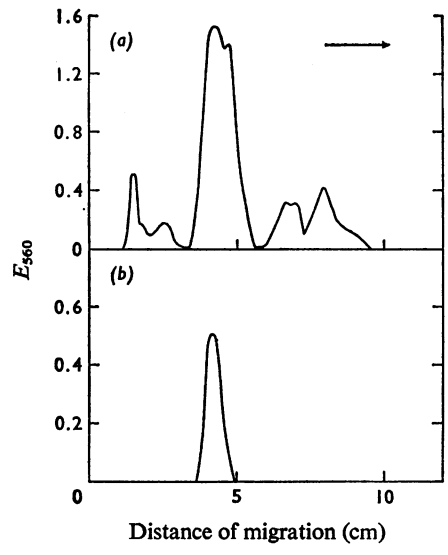

Fig. 9. *Sodium dodecyl sulphate–polyacrylamide-gel electrophoresis showing the purification of actin on Sepharose–myosin*

(*a*) Sample applied to column; (*b*) material eluted by 2mM-Mg·PP_i. Actin was extracted from acetone-dried muscle powder (0.25g) (see the Materials and Methods section) for 30min with 10ml of 2mM-Tris–HCl, pH8.0, containing 0.2mM-ATP, 0.5mM-2-mercaptoethanol and 0.2mM-$CaCl_2$. After filtering through glass wool, the filtrate was dialysed overnight against 1 litre of 50mM-KCl containing 1mM-$MgCl_2$ and 5mM-triethanolamine–HCl, pH7.5. The resulting solution was clarified by centrifuging for 40min at 10000*g* and 4ml applied to a Sepharose–myosin column (6cm×0.9cm) equilibrated with the same buffer. This column was operated as described in the legend to Fig. 8 except that elution of the actin was achieved by including 2mM-sodium pyrophosphate in the above buffer. The ordinate is the intensity of Coomassie Brilliant Blue-stained material, as measured by its E_{560}. The abscissa indicates the distance the proteins have migrated (in cm) from the origin. Migration is from left to right as indicated by the arrow.

the basis of the amount of myosin immobilized to the matrix. The low ATPase activity found in the presence of Mg^{2+} alone is at variance with a report by Liu-Osheroff & Guillory (1972), who attached subfragment 1 covalently to a cellulose ion-exchange matrix and found it to be activated by both Ca^{2+} and Mg^{2+}. These authors propose that the coupling of subfragment 1 to their matrix either provides the enzyme with a new active-site structure or with a new environment resembling in some way that existing on complexing with actin. This indeed may be the case in their system, but coupling the parent molecule to an agarose matrix clearly results in an immobilized myosin which much more closely resembles the molecule in free solution.

The stimulation of the Mg^{2+}-stimulated ATPase activity of Sepharose–myosin by F-actin was generally only two- to three-fold (as opposed to some 50-fold stimulation of the free myosin under these conditions) but this was a reproducible value. This stimulation can be shown to be of a specific nature more clearly in Fig. 8 where a column of Sepharose–myosin has been used to bind F-actin. The bound actin could be eluted from the matrix by irrigation of the column with either ATP or pyrophosphate. Once again, raising the concentration of KCl in the developing buffer to 0.5M also resulted in dissociation of the actomyosin complex. Under these conditions, this immobilized myosin derivative showed no ability to retain non-specific proteins such as bovine serum albumin. Indeed, this matrix could be used to isolate actin directly from extracts of acetone-dried muscle powder (Fig. 9). The fraction eluted by Mg·PP_i (Fig. 9*b*) contained a single component which migrated identically with authentic actin samples.

The Sepharose–myosin conjugate could also be shown to react reversibly with pyrophosphate, ADP and ATP by frontal analysis-type chromatography experiments. This technique has been developed to provide a method for the determination of binding

constants between the immobilized myosin and these small ligands (R. C. Bottomley, A. C. Storer & I. P. Trayer, unpublished work).

Discussion

The special interactions occurring between the various proteins found in the myofibril can be readily exploited in affinity-chromatography experiments. It has been reported from this laboratory (Syska *et al.*, 1974) that the Ca^{2+}-sensitive interaction between two of the components of the troponin complex, the calcium-binding protein (TN–C) and the inhibitory protein (TN–I), can be used to purify TN–I directly from whole muscle homogenates on Sepharose–TN–C conjugates. In the present report we have investigated the possibilities of using the interaction between actin and myosin, the process by which muscle converts chemical energy into mechanical work, in affinity-chromatography experiments as a means of studying this subtle process. Virtually all the work described in this report was performed on immobilized G-actin derivatives since these matrices gave reproducible results. The F-actin matrices, however, rapidly lost much of their binding capacity for subfragment 1, especially when brought into contact with solutions of low ionic strength containing ATP, i.e. typical elution conditions for subfragment 1 or heavy meromyosin bound to Sepharose–actin. The most likely explanation for these observations is that when F-actin is coupled to CNBr-activated Sepharose only relatively few of the actin monomers in the F-actin chains are covalently attached to the matrix. Thus the subsequent initial washing procedures and usage of this matrix in chromatography experiments would be likely to promote a slow (or fast, depending on conditions) depolymerization of the F-actin and a gradual leakage of the non-covalently bound actin from these columns. This would eventually result in a low capacity G-actin matrix, which seems to explain the observed results. Even when washed and operated in the presence of 50mM-KCl, which should slow down this process, the variability of these F-actin matrices was still apparent. The use of Sepharose–F-actin conjugates to study tropomyosin- and troponin-binding has been reported (Kondo *et al.*, 1972) and these workers stated that their F-actin matrices were stable under their conditions of coupling and usage. Nevertheless, in view of our experience we concentrated our efforts on using the Sepharose–G-actin matrix.

Little is known about the interaction between G-actin and myosin; indeed, it has been reported that G-actin will not activate the myosin Mg^{2+}-stimulated ATPase (Tawada & Oosawa, 1969*a*). More recent reports, however, of experiments in which either a kinetic (Offer *et al.*, 1972) or an affinity-chromatography approach has been used (Bottomley & Trayer, 1973; Chantler & Gratzer, 1973) have shown that a small activation does occur. The results presented here also demonstrate a small, but significant, activation of the Mg^{2+}-stimulated ATPase activity of myosin, heavy meromyosin and subfragment 1 by the Sepharose–actin. Offer *et al.* (1972) reported a 1.6-fold activation of the subfragment 1 ATPase activity by G-actin at 25°C (higher at 15°C), whereas in our system an activation of three- to five-fold was observed at 25°C (Fig. 1). Despite the fact that these activations are low compared with those obtained with F-actin, they do serve to confirm that there is enough information in a single actin monomer to cause some enhancement of the ATPase activity and that it is unlikely to be a property shared between two neighbouring actin monomers on the F-actin chains.

The question of whether the G-actin molecule is equivalent to a subunit of F-actin or whether the polymerization process leads to a change in the conformation of the actin (with a resulting ability to interact with myosin etc.) has yet to be answered. The results presented here, both the enzymic studies and more importantly the column chromatography, can be interpreted in this light. The column work clearly establishes the formation of a specific complex between G-actin and heavy meromyosin and subfragment 1 in the absence of ATP. This complex-formation is also suggested by the work of Offer *et al.* (1972) and more clearly indicated by the work of Chantler & Gratzer (1973) and Cooke & Morales (1971). Thus, on the one hand, the immobilized G-actin (like free G-actin) confers only a small enhancement to the Mg^{2+}-stimulated ATPase activity of myosin, showing a diminished activity over that found with the subunits of F-actin, whereas on the other hand, it easily forms a specific complex with the myosin fragments which is readily dissociated by ATP. It is tempting to separate these two properties and consider whether complex-formation necessarily leads to activation of the ATPase activity.

The G-actin monomer does possess all of the capabilities of the F-actin polymer, even though these may not be expressed at full potency. It seems unnecessary, therefore, to postulate that the site on F-actin that interacts with a myosin head is not confined to a single subunit. This subunit of F-actin would then be virtually equivalent to a G-actin monomer. Polymerization to F-actin, as well as being a structural and organizational necessity, would allow the actin subunits to adopt any additional conformational states necessary for full potency of the system to be expressed.

A word of caution should be introduced here. Although steps were taken to ensure that monomeric action was added to the CNBr-activated Sepharose and that the coupling conditions would not normally promote polymerization, no estimation of the state of the covalently bound actin was made. It does seem

unlikely, however, that any polymerization of the G-actin would occur during the coupling procedure resulting in the attachment of (short) polymers to the gel matrix. When different concentrations of G-actin were used in the coupling-stage (in final concentrations ranging from about 0.8 to 4mg/ml), conditions which could conceivably affect the state of polymerization of the actin, no qualitative differences were found in the resulting matrices. This would support the view that the actin was immobilized in the same (G) form. It is also not possible to accurately assess the effects, if any, of the chemical modifications of (some of) the surface lysine residues and of the surrounding monosaccharide matrix on the integrity of the actin molecule. The results expressed in the first part of this report would suggest that these were, at the most, minimal. Additional support for this contention can be obtained from some preliminary experiments which have shown that this Sepharose–actin conjugate can be used to bind troponin-I and, indeed, a specific peptide obtained by fragmentation of this molecule (H. Syska & S. V. Perry, personal communication). Nevertheless, these possible sources of error must be borne in mind when interpreting the data presented in this report.

The specific nature of the interaction between this Sepharose–G-actin conjugate and subfragment 1 and heavy meromyosin has been amply demonstrated. Unfortunately the high ionic strength necessary to maintain the whole myosin molecule in solution has prevented the use of this matrix for studying native myosin–actin interaction. When the columns were operated at an ionic strength greater than 0.25 then no binding of myosin or its fragments to the immobilized actin was observed. Although this could imply interactions of an ionic nature occurring between these proteins, the possibility that the Sepharose–G-actin conjugate was behaving merely as a complex ion-exchange matrix can be eliminated in view of the specificity it has shown. In any case, both proteins would possess a net negative charge at the pH7.5 at which the columns were run. Nevertheless, it is difficult to assess the effect of ionic strength on the myosin–actin interaction. The fact that the activation of the Mg^{2+}-stimulated ATPase of myosin and its fragments by either F-actin or Sepharose–actin was strongly inhibited by increasing the ionic strength (Fig. 1) shows that the myosin–actin interaction must be sensitive to this parameter. The classical experiments of Szent-Gyorgyi (1947) in which the specific viscosity of actomyosin solutions was lowered by increasing the KCl concentration in the absence of ATP also imply dissociation of the protein complex in response to an increase in ionic strength. It has even been suggested that the interaction of myosin and actin at high ionic strength may be of a different type from that existing under physiological conditions (see review, Perry, 1967). The results presented here clearly indicate that the strength of the myosin–actin interaction, as demonstrated in this affinity-chromatography system, is lowered as the ionic strength is increased, particularly in the absence of ATP.

At first sight, these data are at variance with the report by Eisenberg *et al.* (1972) who found that the binding of heavy meromyosin to F-actin, in the absence of ATP was unaffected by ionic strength over the range 0.01–0.1 mol/l. Steady-state kinetic studies by Eisenberg & Moos (1968, 1970) on the F-actin–heavy meromyosin Mg^{2+}-stimulated ATPase in which they showed that this was inhibited by KCl concentrations as low as 20mM can readily be reconciled with our results. The explanation for the differences observed between their observations in the absence of ATP and ours almost certainly lies in the actin used. These workers were using F-actin where it is possible for both heads of the heavy meromyosin molecule to bind simultaneously to the F-actin in the absence of ATP, whereas this is a very unlikely situation in our system. This could result in a reinforced binding of heavy meromyosin to F-actin in the absence of ATP which is not possible in its presence. In this respect it was noteworthy that we were unable to successfully fractionate subfragment 1 and heavy meromyosin on our G-actin matrices. Gradient elution of these proteins, in separate experiments, from Sepharose–actin suggested that the heavy meromyosin may bind slightly more strongly but no resolution of an applied mixture was obtained under the conditions used. Although additional data are required before any firm conclusions can be reached, these observations are consistent with a model in which each actin subunit can interact with a single myosin head and at least in terms of this G-actin–single-myosin-head system both heads of the parent molecule behave equivalently and independently.

Chemical modification of two of the thiol groups (per subunit) in the head region of the myosin molecule has frequently been used as a means of studying myosin–actin–ATP interactions. Myosin or its fragments in which these groups have been either partially or fully labelled still possess the ability to interact strongly with both the Sepharose–G-actin and 6-aminohexan-1-ol pyrophosphate derivatives showing the accessibility of these sites on the modified molecule, even on those in which the enzymic activity has been totally destroyed. These observations are supported by some of the recent modification studies on these myosin thiol groups. Malik & Martonosi (1972) found that ADP would still bind to *N*-ethylmaleimide-inactivated heavy meromyosin. The selective attachment of spin labels to either the $-SH_1$ or $-SH_2$ groups have shown that these labels undergo spectral changes during ATP-binding and hydrolysis (Seidel & Gergely, 1972, 1973) and on actin-binding (Tokiwa, 1971; Seidel, 1973). Apart from the rapidity and simplicity of the affinity-chromatography approach

in evaluating the result of the modifications, the sensitivity of the method in comparing binding abilities is shown in Fig. 6.

The use of gradient elution techniques greatly extends the potential of this methodology. As expected, ATP proved more efficient at dissociating the immobilized actin–subfragment 1 complex than did ADP (which was as good as pyrophosphate). These observations are entirely consistent with the kinetic model for the hydrolysis of ATP by actomyosin proposed by Lymn & Taylor (Taylor *et al.*, 1970; Lymn & Taylor, 1970, 1971). The dissociation of actomyosin by ATP is seen here as a much faster process than the dissociation of this complex by ADP, some 10 times faster according to the kinetic constants given by Lymn (1973). The binding of Mg·ATP to actomyosin results in the dissociation of this complex followed by the rapid hydrolysis of ATP to form a myosin–products complex. Such a complex could recombine with Sepharose–actin resulting in a rapid release of the products and the cycle repeated as the subfragment 1 percolates through the column. This would help explain both why the elution by Mg·ATP did not occur at as low a concentration as would be expected from the observed rate constants of the process and why the subfragment 1 was eluted as a broad peak. The more efficient elution observed by ATP in the absence of any bivalent cations could then be explained by this nucleotide effecting a dissociation of the immobilized protein complex with no subsequent hydrolysis.

The covalent attachment of myosin to an agarose matrix can also be used as a model system for investigating the properties of this system. Myosin immobilized in this manner possesses all the properties, although these are diminished, of the molecule in free solution. Only very low activations of the Mg^{2+}-stimulated ATPase activity by F-actin were apparent but this may well be due to the gel matrix limiting the diffusion of this large polymer. Since the ability of myosin and heavy meromyosin to promote polymerization of G-actin is well documented (Tawada & Oosawa, 1969*b*; Kikuchi *et al.*, 1969), activation by the monomeric species was not tried. A specific interaction between the immobilized myosin and F-actin was demonstrated chromatographically, however (Fig. 8), and the myosin columns were capable of isolating actin direct from extracts of acetone-dried muscle powder. This affinity-chromatography technique is unlikely to find much application in the preparation of skeletal muscle actins for which adequate and simple methods exist. Actin and actin-like molecules, however, have been shown to exist in a variety of non-muscle cells and direct isolation of actin from these tissues on a myosin matrix, perhaps as some form of screening procedure, is an attractive possibility.

Thus the immobilized actin and myosin derivatives offer a new parameter for studying myosin–actin interactions. The specificity of this interaction can be readily used in purification methodology and can provide a rapid and simple technique for evaluating the results of chemical modification experiments.

We gratefully acknowledge the financial support given by the Science Research Council for this project. R. C. B. is supported by a Medical Research Council Training Award.

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