Biochem. J. (1972) **127**, 419–424
Printed in Great Britain

The Enzymic Properties of a Modified Ox Heart Myosin Adenosine Triphosphatase on Covalent Binding to an Insoluble Cellulose Matrix

By PHYLLIS LIU-OSHEROFF and RICHARD JOHN GUILLORY*
Section of Biochemistry and Molecular Biology, Cornell University, Ithaca, N.Y. 14850, *U.S.A.*

(*Received* 5 *October* 1971)

The preparation of ox heart myosin and its partial digestion with cellulose-bound papain is described. A procedure is outlined by which heavy meromyosin subfragment 1 can be covalently bound to a cellulose ion-exchange matrix. Attachment of heavy meromyosin subfragment 1 to the insoluble matrix results in a change in the ion specificity towards ATP hydrolysis. Unlike the soluble enzyme the bound form is activated by both Ca^{2+} and Mg^{2+}. Maximal activation by Ca^{2+} occurred at a lower concentration for the bound enzyme. Mg^{2+} activates at a concentration which causes near-maximal inhibition of the Ca^{2+}-activated adenosine triphosphatase (ATPase) of the non-bound enzyme. The Mg^{2+}-activated ATPase of the bound enzyme was in turn inhibited by the presence of Ca^{2+}. The activation by Mg^{2+} resembles the characteristic enzymic action of the actin-subfragment 1 complex.

The attachment of enzymes to water-insoluble supports is now the subject of interest from both a theoretical and practical point of view (Silman & Katchalski, 1966; Crook, 1968; Manecke, 1968; Gabel & Hofsten, 1970; Glassmeyer & Olge, 1971). From the standpoint of enzymology the most satisfactory method developed to date for such binding has been covalent linking of proteins to polymeric substances via protein functional groups which are non-essential for biological activity. A natural prerequisite for such linking reactions is the maintenance of low degrees of protein denaturation. Cellulose, having hydroxyl groups available for the introduction of various reactive functional sites, has been found to be a suitable natural substance for interaction with proteins (Manecke, 1968). At the present time the preparation of matrix-bound enzymes is confined primarily, although not exclusively, to hydrolytic enzymes (Crook, 1968). Our interest in such modified proteins rests in the theoretical possibility that these derivatives may provide relatively simple models with which to study the effect of changes in the microenvironment of the active site. This is of greatest interest for those enzymes or other biologically active proteins which act *in vivo* while being embedded within a membrane structure.

It is well known that the interaction of the two major muscle proteins, myosin and actin, brings about a change in the ion specificity of the myosin ATPase† activity. Myosin ATPase is activated by Ca^{2+} and inhibited by Mg^{2+}, whereas the actomyosin complex is Mg^{2+}-activated (Green & Mommaerts, 1953). Heavy meromyosin subfragment 1, the globular portion of the myosin molecule isolated by digestion with either trypsin or papain (Kominz *et al.*, 1965; Lowey *et al.*, 1969; Tada *et al.*, 1969), retains the actin-combining and ATPase properties of the parent molecule. The complexing of actin with subfragment 1 leads to an ATPase activity which is stimulated by Mg^{2+} at low ionic strength (Eisenberg *et al.*, 1968).

It is possible that this alteration in ion specificity for ATPase activation may imply certain fundamental changes in the microenvironment of the myosin molecule at the active site and the question arises as to whether actin is specifically required for such changes.

The present paper reports on the attachment of enzymically active subfragments of ox heart myosin to diazotized *p*-aminobenzylcellulose ion exchanger. Examination of the ATPase properties of the resulting insoluble enzyme–cellulose complex showed that binding caused certain changes in the enzymic characteristics of the subfragment 1 ATPase. From these studies it is concluded that the changed ion specificity of myosin ATPase on interaction with actin could be an intrinsic property of the myosin ATPase site, independent of any specific interaction with actin.

Methods

Preparation of myosin

Myosin was prepared from ox heart muscle. All procedures were carried out in the cold with the use of glass-distilled water. Coarsely minced heart muscle (approx. 300g) was washed twice with

* Present address: Department of Biochemistry and Biophysics, University of Hawaii, Honolulu, Hawaii 96822, U.S.A.

† Abbreviations: ATPase, adenosine triphosphatase; PAB-cellulose, *p*-aminobenzylcellulose.

500ml of 0.02M-EDTA, pH7.4, followed by two washings with 500ml of glass-distilled water. Each time the wash solution was separated from the muscle pulp by straining through two layers of cheese-cloth. The washed pulp was extracted with 3vol. of 0.3M-KCl–0.1M-KH_2PO_4–0.05M-K_2HPO_4–0.5mM-ATP, pH6.7, for no more than 20min. The final extract was clarified by centrifugation for 20min at 12100*g* and diluted to 4vol. with 0.5mM-ATP, pH6.7, and immediately strained through cheese-cloth into a 6-litre cylindrical vessel. A solution of 0.5mM-ATP was then added slowly up to a total volume equivalent to 12vol. of original solution. The protein precipitate was allowed to settle for 1h. Most of the supernatant was siphoned off with a water aspirator and the precipitate collected by centrifugation at 12100*g* for 30min. The protein was then dissolved in 90ml of 2M-KCl–0.1M-tris–HCl (pH6.7)–0.5mM-ATP, and clarified by centrifugation at 110000*g* for 1h. The protein solution was diluted with 0.5mM-ATP, pH6.7, to an ionic strength of 0.5 and then clarified by centrifugation for 30min at 80000*g*. The supernatant was further diluted to an ionic strength of 0.25, causing the precipitation of actomyosin, which was removed by centrifugation at 12100*g* for 15min. The supernatant was finally diluted slowly to an ionic strength of 0.037, causing the precipitation of myosin, and the precipitate was collected by centrifugation at 12100*g* for 30min. The pellet was dissolved in 25ml of 2.0M-KCl–0.1M-tris–HCl (pH6.7)–0.5mM-ATP, and clarified by centrifugation for 1h at 110000*g*.

Determination of protein

Protein was determined by the biuret method (Gornall *et al.*, 1949) in a total volume of 2.5ml by using human serum albumin as the standard.

ATPase assay

ATPase assays were carried out at 30°C. The 1ml incubation mixture usually contained 40mM-tris–HCl buffer, pH8.0; 40mM-$CaCl_2$; 5mM-ATP; and protein (0.05–0.3mg). The reaction was initiated by the addition of ATP and stopped by addition of 1ml of 15% (w/v) trichloroacetic acid. The protein precipitate was sedimented and the protein-free supernatant assayed for P_i by the method of Lohman & Jendrassik (1924) scaled down to a 5ml volume. The specific activity of the ATPase was evaluated as μmol of P_i liberated/10min per mg of protein.

Preparation of insoluble papain

Water-insoluble papain was prepared by coupling papain to diazotized PAB-cellulose ion exchanger essentially as described by Lowey *et al.* (1969). Serva PAB-cellulose ion exchanger (Gallard–Schlesinger Chemical Manufacturing Corp., Carle Place, N.Y., U.S.A., 105no. C. 3014, 0.3mequiv./g) was suspended in 30ml of 50% (v/v) acetic acid. Sufficient water was added to make a slurry mixture, which was then acidified with 5ml of 2M-HCl and chilled to ice temperature. Diazotization of the cellulose was carried out by dropwise addition of ice-chilled 0.5M-$NaNO_2$ (about 2.5ml) to the well-mixed suspension until the colour changed from rusty brown to pastel orange. The mixture was left on ice in a loosely stoppered vessel. After approx. 3h the pH was slowly raised to 7 with cold 50% (w/v) NaOH (the temperature was at all times kept close to 0°C) and finally adjusted to pH8 with cold 2M-NaOH. The suspension was filtered on a Buchner funnel with the aid of a water aspirator and the filtered cake washed successively with 150ml of cold 10% (w/v) sodium acetate and 150ml of cold 0.1M-sodium phosphate buffer, pH7.5, and finally suspended in 100ml of 0.075M-sodium phosphate–5mM-cysteine–2mM-EDTA, pH 7.5. Coupling with papain was carried out by stirring 3ml of a suspension of crystalline papain in 0.05M-sodium acetate, pH4.5 (Worthington Biochemical Corp.; lot no. PAP 9KA; 30mg/ml, 0.12 unit/mg) together with 10ml of 0.03M-cysteine, pH7.5, into the diazotized cellulose suspension. The mixture was flushed with N_2 and stirred overnight in a tightly stoppered flask. No diazonium groups could be detected with α-naphthol in 10% (w/v) NaOH (red colour develops for positive test). The suspension was washed several times on a Buchner funnel with a total of 400–500ml of cold 5mM-cysteine–2mM-EDTA, pH6.0. The insoluble papain was finally suspended in 60ml of 5mM-cysteine–2mM-EDTA, pH6.0, flushed with N_2 and stored at 4°C. Such preparations were used for no more than 3 weeks.

Assay of insoluble papain

The enzymic activity of insoluble papain was determined by pH-stat titration of the acid produced during the hydrolysis of *N*-benzoyl-L-arginine ethyl ester hydrochloride (Cyclo Chemical Corp., Los Angeles, Calif., U.S.A., lot no. K-5647) at 25°C, pH6.2, by using a Radiometer TTT-1 titrator essentially as described in the Worthington Biochemical Corporation's publication 'Enzymes' section 3.4.410. The substrate was prepared by mixing 592mg of *N*-benzoyl-L-arginine ethyl ester hydrochloride in 30ml of water with 1.6ml of 0.01M-EDTA and 1.6ml of 0.05M-cysteine (freshly prepared), adjusting the pH to 6.2 and the final volume to 42ml. The test mixture contained 2.5ml of the substrate, 2.5ml of 3M-NaCl, appropriate amount of insoluble papain suspension and water in a total volume of 8ml. The pH of the reaction mixture was maintained at 6.2 by automatic titration with 0.01M-NaOH.

A unit of enzyme activity is defined as that quantity of cellulose-bound papain which hydrolyses 1 μmol of *N*-benzoyl-L-arginine ethyl ester hydrochloride/min. pH-stat measurements of the enzymic activity of insoluble papain showed that within the concentration range 12–62 μg of protein/ml of reaction mixture the activity averaged 6.8 μmol/min per mg of enzyme.

Digestion of myosin with insoluble papain

Digestion of myosin with insoluble papain was performed by incubating a 2.4% (w/v) solution of myosin in 2M-KCl–0.1M-tris–HCl buffer, pH6.7, with 0.33mg of insoluble papain/ml (final concentration) and 0.5ml of 0.3M-cysteine–0.2M-EDTA, pH7.5, for 20min at 25°C. The mixture was then centrifuged for 30min at 27000*g* at 0–4°C. The insoluble papain was completely removed by filtration of the supernatant through Whatman no. 1 filter paper previously washed extensively with 2mM-EDTA, pH7.5. The digest was dialysed overnight against 6 litres of 5mM-tris–HCl buffer, pH6.7, containing 0.2mM-ATP. A precipitate formed as a result of the dialysis was sedimented by centrifugation for 30min at 27000*g* followed by a second 30min centrifugation at 80000*g*. The resulting supernatant is the source of the myosin subfragment (Lowey *et al.*, 1969). The sedimented protein from the two centrifugations was dissolved in 10ml of 2M-KCl–0.1M-tris–HCl buffer, pH6.7, and clarified by centrifugation for 20min at 27000*g*. All fractions were assayed for protein and ATPase activity.

Coupling of the papain-digested myosin to the cellulose ion-exchange derivative

The soluble fraction isolated from the papain-digested myosin (most probably a mixture of subfragment 1 and subfragment 2; Lowey *et al.*, 1969) was coupled to diazotized PAB-cellulose ion exchanger in a manner essentially similar to the procedure used for the preparation of the insoluble papain derivative. An 8ml solution of the papain-digested myosin containing about 30mg of protein was stirred overnight with the diazotized cellulose suspended in 100ml of 0.075M-sodium phosphate–5mM-cysteine–2mM-EDTA, pH7.5. The resulting insoluble complex was then filtered on a Buchner funnel and washed with 500ml of 5mM-cysteine–2mM-EDTA, pH7.5, followed by 200ml of 0.1M-tris–HCl buffer, pH7.0, and was finally suspended in 60ml of 0.1M-tris–HCl buffer, pH7.0.

pH-stat measurements

Ca^{2+} or Mg^{2+}-activated ATPase activities were measured by means of pH-stat titration of the acid released during the hydrolytic cleavage of ATP at 25°C (Green & Mommaerts, 1953). The reaction mixture, in a total volume of 2ml, contained 1ml of the insoluble protein–cellulose suspension, 3mM-tris–H_2SO_4 buffer, pH7.4, 15mM-ATP, pH6.7, and the amounts of $CaCl_2$, $MgCl_2$ or KCl as indicated for the individual experiments. The reaction was initiated by the addition of ATP after a 2min preincubation with enzyme to allow for temperature equilibration. Since the amount of acid liberated per mol of ATP hydrolysed is a function of the pH (Green & Mommaerts, 1953), the final pH at which the reaction was carried out (pH6.5–6.9) was taken into account in the calculation of enzymic activities. After each determination the reaction mixture was collected in a small pre-weighed test tube and centrifuged. Most of the supernatant was discarded and the insoluble protein–cellulose complex was dried in an oven at 100°C, cooled in a desiccator and weighed. The drying and weighing were repeated until constant dry weight was obtained. A 1–9% difference was obtained for the dry weights of all the samples. ATPase activity was expressed as μmol of P_i liberated/min per g dry weight of the protein–cellulose complex.

Table 1. *Solubilization of ox heart myosin protein by papain digestion*

The concentration of myosin during papain digestion, the digestion period and the final concentration of insoluble papain used in the digestion mixture were: preparation 1; myosin, 0.35%; 10min; papain, 0.15mg/ml; preparation 2; myosin, 2.4%; 20min; papain, 0.33mg/ml. The soluble fraction is that obtained by digestion of myosin with insoluble papain, followed by dialysis against 6 litres of 5mM-tris–HCl buffer (pH6.7)–0.2mM-ATP.

	Myosin		Soluble fraction		
Preparation	Protein (mg/ml)	Total protein (mg)	Protein (mg/ml)	Total protein (mg)	% of myosin protein solubilized by papain
1	10.3	103	3.3	46.2	44.8
2	24.3	580	7.2	259.0	44.6

Table 2. *ATPase activities of myosin and the soluble and insoluble fractions of papain-digested myosin*

The digestionconditions are the same as those outlined for Table 1. The procedures used for the isolation of the soluble and insoluble fractions are described in the Methods section.

	ATPase activity (μmol of P_i liberated/10min per mg of protein)			
		Soluble fraction	Insoluble fractions	
Preparation	Myosin		I	II
1	3.30	8.80	0.84	0.88
2	5.20	10.06	0.45	0.00

The ATPase activity of soluble subfragment 1 obtained by direct determination of the phosphate liberated from ATP was found to be identical with that calculated by using the pH-stat titration method.

Results

Digestion of myosin with insoluble papain

Protein determination and ATPase assays for the soluble and insoluble fractions of the papain-digested myosin after dialysis against a low-ionic-strength buffer showed that the amount of protein recovered in the soluble fraction was about 45% of the total myosin protein (Table 1). The ATPase activity of the soluble fraction, however, was about 1.9–2.6 times that of the parent myosin molecule and about 10–22 times that of the insoluble fractions (Table 2). This agrees with the report by Lowey *et al.* (1969) that the soluble fraction resulting from complete papain digestion of myosin contained a mixture of subfragment 1, the enzymically active, globular subunit of myosin and subfragment 2, the helical portion of heavy meromyosin. The present investigation does not eliminate the possibility of incomplete digestion, in which case a small amount of heavy meromyosin might also be present in the soluble fraction. Subfragments 1 and 2 and heavy meromyosin are all known to be soluble at low ionic strength. The insoluble fraction isolated at low ionic strength from effective papain digestion would consist only of light meromyosin. If digestion was not completely effective, in addition to light meromyosin, myosin rods (i.e. myosin without the two enzymic ATPase sites) and modified myosin (i.e. myosin that had retained only one of its two globular portions) might all be present in the insoluble fractions. The presence of such modified myosin species might account for the ATPase activity exhibited by the low-ionic-strength-precipitable protein after papain digestion (Table 2).

Diazo coupling to cellulose ion-exchange derivative of the soluble myosin fraction after papain digestion

Only a minor amount of ATPase activity could be detected in the washings of the papain-digested myosin–cellulose couple, indicating a high degree of coupling of the subfragment to the cellulose derivative. If the final protein–cellulose complex was suspended in 5mM-cysteine–2mM-EDTA, pH7.5, the preparation was stable for some 3 days, losses in activity perhaps being caused by the oxidation of cysteine. Although the preparation was flushed very frequently with N_2 it was often under aerobic conditions especially during sampling in preparation for analysis. An alternative explanation for the inactivation in cysteine–EDTA at pH7.5 could be the formation of mixed disulphides between the protein–cellulose complex and cysteine. To circumvent this inactivation an additional wash of the enzyme matrix was carried out with 0.1M-tris–HCl buffer, pH7.0, after the cysteine–EDTA wash, and the complex was then suspended in 0.1M-tris–HCl buffer, pH7.0. In this way the enzyme activity was stabilized for periods in excess of 2 weeks.

Enzymic characteristics of the cellulose-bound ATPase enzyme

A number of enzymic characteristics of the matrix-bound subfragment ATPase were found to differ from those of the soluble enzyme. The non-bound subfragment ATPase is known to be Ca^{2+}-activated. In the present study the ATPase activity of the soluble subfragment 1 was found to be maximal and reached a plateau at 40–80mM-Ca^{2+} (Fig. 1). The presence of Mg^{2+} at low concentrations significantly inhibited the Ca^{2+}-activated ATPase activity of this protein (Fig. 2). On the other hand, it was shown that maximal activation of the ATPase activity of the cellulose-bound derivative occurred at about 5mM-Ca^{2+}, and that Mg^{2+} at 2.5mM, a concentration that brought about maximal inhibition of the non-bound

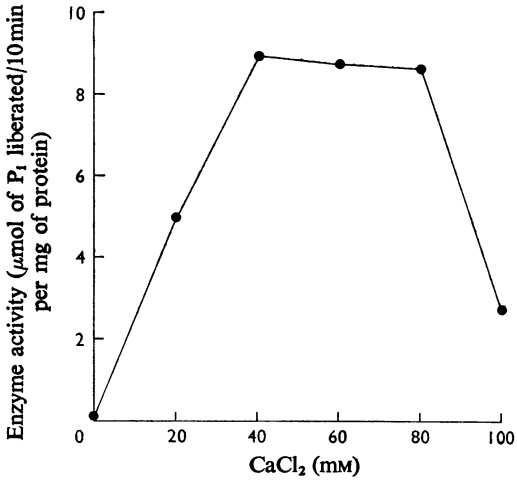

Fig. 1. *Ca^{2+} activation of subfragment* 1 *ATPase*

The ATPase assay mixture in a total volume of 1ml contained: 40mM-tris–HCl buffer, pH8.0; 5mM-ATP; 0.07mg of subfragment 1 and $CaCl_2$ as shown. P_i liberated after the 10min incubation at 30°C was determined by the method of Lohman & Jendrassik (1924).

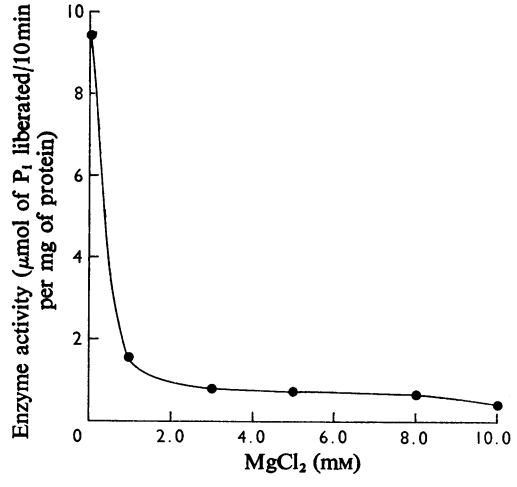

Fig. 2. *Effect of low concentrations of Mg^{2+} on the Ca^{2+}-activated ATPase of subfragment* 1

Assay conditions were as for Fig. 1 except that $CaCl_2$ remained constant at 40mM final concentration in the assay mixture and $MgCl_2$ concentrations were varied as shown.

preparation, activated the ATPase activity of the bound form (Fig. 3). In addition the concentration dependence and the maximal activation of the activity were similar for both Ca^{2+} and Mg^{2+}.

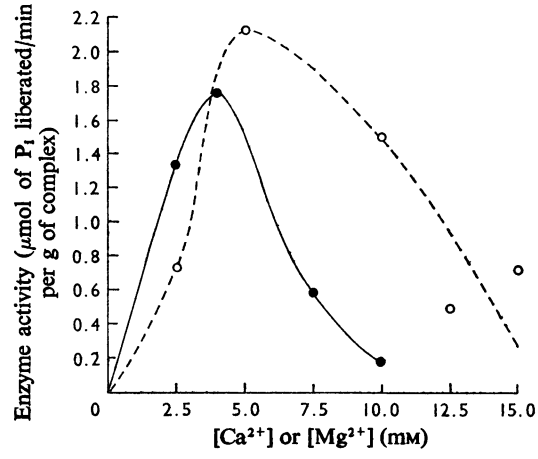

Fig. 3. *Ca^{2+} (o) and Mg^{2+}(●) activation of the ATPase of subfragment* 1 *bound to the cellulose derivative*

The ATPase pH-stat assay mixture in a total volume of 2ml contained 3mM-tris–H_2SO_4 buffer, pH7.4; 15mM-ATP, pH6.7; 1ml of the protein–cellulose complex (dry weight determined after the assay); and $CaCl_2$ and $MgCl_2$ as shown. Assays were carried out at 25°C.

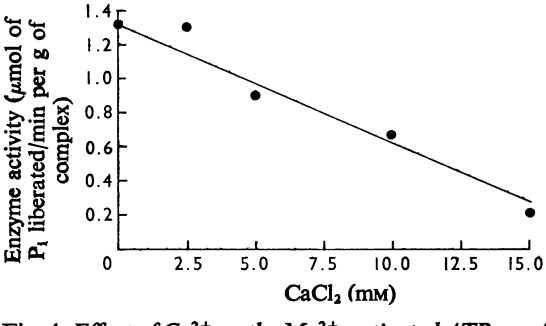

Fig. 4. *Effect of Ca^{2+} on the Mg^{2+}-activated ATPase of subfragment* 1 *bound to the cellulose derivative*

Assay conditions were as for Fig. 3 except that $MgCl_2$ remained constant at 2.5mM final concentration in the assay mixture and $CaCl_2$ concentrations were varied as shown.

The activation of the bound enzyme by Mg^{2+} could not be accounted for solely on the basis of the ionic strength since lower activation (μmol of P_i liberated/min per g dry wt. of protein-cellulose complex) was obtained in the presence of 15mM-KCl (0.589μmol of P_i/min per g dry wt.), and only a slightly higher activation was obtained when Mg^{2+} (2.5mM) and KCl (15mM) were present together (compare 1.322μmol of P_i liberated with Mg^{2+} alone

with 1.698 with Mg^{2+} and KCl together). Maximal activation by Mg^{2+} occurred at 4mM (Fig. 3). This Mg^{2+}-activated ATPase activity of bound subfragment 1 decreased with increasing concentrations of Ca^{2+} (Fig. 4). This inhibition may be due to the presence of increasing concentrations of bivalent ions analogous to the influence of high concentrations of Ca^{2+} or Mg^{2+} (Fig. 3), or alternatively to the possibility that the Mg^{2+}-activated ATPase activity of the bound enzyme is inhibited specifically by Ca^{2+}.

Discussion

Results of the present study show that the formation of an insoluble complex between heavy meromyosin subfragment 1 and a cellulose derivative alters the ion specificity and optimum ion concentration for maximal activation of subfragment ATPase. Whereas non-bound subfragment ATPase is known to be Ca^{2+}-activated, the bound subfragment activity has been shown to be activated by both Ca^{2+} and Mg^{2+} to a similar extent and over a similar concentration range. The maximal activity of non-bound subfragment ATPase occurred at 40mM-Ca^{2+}, whereas that of the bound subfragment ATPase occurred at 5mM-Ca^{2+} or 4mM-Mg^{2+}. The maximal activation of myosin ATPase, on the other hand, is at 20mM-Ca^{2+}.

There thus appears to be a loss in Ca^{2+} sensitivity towards myosin ATPase activation on digestion with papain. This sensitivity is then regained and intensified when the protein is bound to the cellulose matrix; however, the ion selectivity of the ATPase activity is lost.

The present work shows that Mg^{2+} at 2.5mM, a concentration that almost completely inhibits the Ca^{2+}-activated ATPase of non-bound subfragment 1, activates the bound form of the enzyme and that this Mg^{2+}-activated ATPase is inhibited by higher concentrations of Mg^{2+} itself or by Ca^{2+}.

The influence of Mg^{2+} on the ATPase activity of cellulose-bound subfragment 1 resembles in some ways the characteristics of an actin–subfragment 1 complex in that both types of preparations are activated by Mg^{2+} at low ionic strength (Eisenberg *et al.*, 1968). It is proposed that attachment of heavy meromyosin subfragment 1 to the cellulose derivative via covalent bonds provides the enzyme with a new active-site structure or environment resembling in some ways that formed on complexing with actin. This alteration in the ion specificity of the ATPase activity on binding to the cellulose matrix demonstrates that ion specificity can be a characteristic of the myosin molecule independent of an interaction with actin.

R. J. G. is an Established Investigator of the American Heart Association.

References

Crook, E. M. (1968) *Biochem. J.* **107**, 1P
Eisenberg, E., Zobel, C. R. & Moos, C. (1968) *Biochemistry* **7**, 3186
Gabel, D. & Hofsten, B. J. (1970) *Eur. J. Biochem.* **15**, 410
Glassmeyer, C. K. & Olge, J. D. (1971) *Biochemistry* **10**, 786
Gornall, A. G., Bardawill, C. S. & David, M. M. (1949) *J. Biol. Chem.* **177**, 751
Green, I. & Mommaerts, W. F. H. M. (1953) *J. Biol. Chem.* **202**, 541
Kominz, D. R., Mitchell, E. R., Nihei, T. & Kay, C. M. (1965) *Biochemistry* **4**, 2373
Lohman, K. & Jendrassik, L. (1924) *Biochem. Z.* **178**, 419
Lowey, S., Slayter, H. S., Weeds, A. G. & Baker, H. (1969) *J. Mol. Biol.* **42**, 1
Manecke, G. (1968) *Biochem. J.* **107**, 2P
Silman, I. H. & Katchalski, E. (1966) *Annu. Rev. Biochem.* **35**, 873
Tada, M., Bailiu, G., Barany, K. & Barany, M. (1969) *Biochemistry* **8**, 4842