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PREPARATION AND CHARACTERIZATION OF AN ENZYMATICALLY ACTIVE IMMOBILIZED DERIVATIVE OF MYOSIN

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Summary

Purified skeletal muscle myosin (EC 3.6.1.3) has been covalently bound to Sepharose 4B by the cyanogen bromide procedure. The resulting complex, Sepharose-Myosin, possesses adenosine triphosphatase activity and is relatively stable for long periods of time. Under optimal binding conditions, approximately 33% of the specific ATPase activity of the bound myosin is retained. Polyacrylamide gel electrophoresis of polypeptides released from denatured Sepharose-Myosin indicates that 85% of the myosin is attached to the agarose beads through the heavy chains and the remainder through the light chains, in agreement with predictions of binding and release based upon either the lysine contents or molecular weights of the myosin subunits. The adenosine triphosphatase of the immobilized myosin has been investigated under conditions of varying pH, ionic strength, and cation concentration. The ATPase profiles of immobilized myosin are quite similar to those for free myosin, however subtle differences are found. The Sepharose-Myosin ATPase is not as sensitive as myosin to alterations in salt concentration and the apparent K_M is approximately two-fold higher than that of myosin. These differences are probably due to chemical modification in the region of the attachment site(s) to the agarose beads and hydration and diffusion limitations imposed by the polymeric agarose matrix.

Introduction

Myosin (EC 3.6.1.3, ATP phosphohydrolase) the major component of the thick filament of muscle, plays a central role in muscle contraction and has

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been extensively investigated (for reviews, see refs 1 and 2). A problem encountered in the in vitro study of myosin is that it is soluble only at high ionic strength and forms filaments and insoluble aggregates at physiological salt concentrations and lower. These solubility characteristics are conferred upon myosin by the fibrous tail portion of the molecule [3]. To study the enzymatic and actin binding properties of myosin under a variety of conditions, it would be advantageous to maintain a constant solubility state and thus eliminate the effects of aggregation.

One approach has been the partial hydrolysis of myosin with the proteolytic enzymes trypsin and papain. Of the digestion products, heavy meromyosin and heavy meromyosin Subfragment 1 possess adenosine triphosphatase (ATPase) activity and retain the ability to bind actin. These fragments are soluble over a wide range of salt concentrations and have been used extensively in the study of the myosin ATPase. Although heavy meromyosin and Subfragment 1 are generally accepted as model systems, one cannot be certain that the proteolytic digestion has not altered the enzymatic properties of the molecule. During the preparation of HMM approximately 20 to 60 bonds are broken [4] and some small peptides are released [5].

Another approach to the solubility problem has been the preparation of a soluble derivative of myosin, poly-alanyl-myosin [6]. Polyalanylation renders myosin soluble in dilute salt solutions, presumably by alteration of charge distribution. However, much of the alteration in the physical and enzymatic properties of this derivative can be ascribed to the exposure of myosin to dioxane during preparation.

Immobilization of myosin onto an insoluble matrix also prevents aggregation. Myosin covalently bound to diazotized *p*-aminobenzyl-cellulose has been used as an immunoadsorbent [7], but its enzymatic properties have not been examined. A mixture of heavy meromyosin Subfragments 1 and 2 has been bound to this ion exchange-cellulose and the ion specificity of the immobilized ATPase has been described [8]. These enzymatic analyses were performed at very low ionic strength, and under these conditions the influence of the supporting matrix on the microenvironment could be significant since large shifts in pH optima and considerable losses of biological activity [9,10] have been reported for many immobilized enzyme systems. These factors were not taken into account in the study of the *p*-aminobenzyl-cellulose derivative.

In order to make use of an immobilized myosin as a model for the myosin ATPase under conditions in which myosin normally aggregates, it is necessary to define conditions which minimize the loss of enzymatic activity and to determine the effects of the supporting matrix on the microenvironment of the enzyme. Investigations were carried out to ascertain these conditions and effects. For these purposes we have covalently bound highly purified myosin to agarose beads. The resulting complex, Sepharose-Myosin, possesses an ATPase activity similar to myosin. Sepharose-Myosin is also able to bind actin in affinity chromatography experiments [11,12].

This paper describes methods for the preparation of Sepharose-Myosin and some of the structural properties of myosin which have been determined from analysis of the immobilization. We also present data regarding the activity of the Sepharose-Myosin ATPase. For the most part, the activity of immobi-

lized myosin under various conditions closely parallels that of unbound myosin. The differences observed may be attributed to either the immobilization process or to the prevention of aggregation. We have attempted to differentiate between these two effects.

The major advantage of Sepharose-Myosin as a model system is the elimination of myosin's solubility properties. Recent evidence suggests that myosin may be associated with the plasma membrane in non-muscle cells [13,14]. If so, Sepharose-Myosin may be a more representative model than "soluble" myosin for these cases.

Materials and Methods

Preparation of proteins

Myosin was extracted [15] from the breast muscle of freshly killed White Leghorn laying hens and further purified by ammonium sulfate precipitation [16] and DEAE Sephadex chromatography in 0.04 M sodium PPi [17]. Actin was extracted from an acetone powder by the method of Spudich and Watt [18]. Protein content was determined by the biuret assay.

Preparation of Sepharose-Myosin

The procedure for protein immobilization was based upon that of Porath et al. [19] as described by Cuatrecasas and Anfinsen [20]. Sepharose 4B (Pharmacia Fine Chemicals) was activated with 250 mg cyanogen bromide (CNBr) per ml of agarose beads. During activation, pH was maintained at 11 by manual titration with 5 M NaOH and the temperature was held constant at 20° C. Upon cessation of base uptake the activated agarose was washed with at least 20 vol. of cold H_2O. Myosin was coupled to CNBr-activated agarose under a variety of conditions using the following coupling buffers: Buffer I, 0.5 M KCl, 0.05 M citrate, 0.01 M Na_4PP_i; Buffer II, 0.5 M KCl, 0.05 M Na_4PP_i; Buffer III, 0.5 M KCl, 0.05 M borate, 0.01 M Na_4PP_i; Buffer IV, 0.5 M KCl, 0.05 M phosphate; Buffer V, 0.05 M KCl, 0.05 M bicarbonate. Prior to the addition of myosin, the H_2O-washed CNBr-agarose was further washed with 20 vol. of cold coupling buffer at varying pH's. Myosin (6—10 mg myosin per ml agarose), previously equilibrated with the buffer was mixed with activated agarose. The mixture was stirred gently in the cold for 16—24 h. In determining the pH coupling curve (Fig. 1) the Sepharose activated with CNBr was divided into 4 ml aliquots and each was washed and equilibrated with a coupling buffer of different pH (buffers I—V). 2 ml of a stock solution of myosin (7.9 mg/ml) in 1 M KCl was added to each sample and the total volume adjusted to 9 ml (final conditions given in Fig. 1). Myosin was reacted with the CNBr-agarose for 45 h at 4° C in rotating tubes. Free myosin was removed by several cycles of washing (on a glass Buchner funnel) with the coupling buffer followed by several washes with buffer II adjusted first to pH 6.2 and later to pH 8.0. Activated groups remaining on the Sepharose-Myosin were blocked by incubation for 2 h at 4° C in a solution containing 0.5 M ethanolamine, 0.05 M Na_4PP_i, pH 8.0. The conjugate was washed free of ethanolamine and suspended in the desired buffer.

All further experiments were carried out with Sepharose-Myosin prepara-

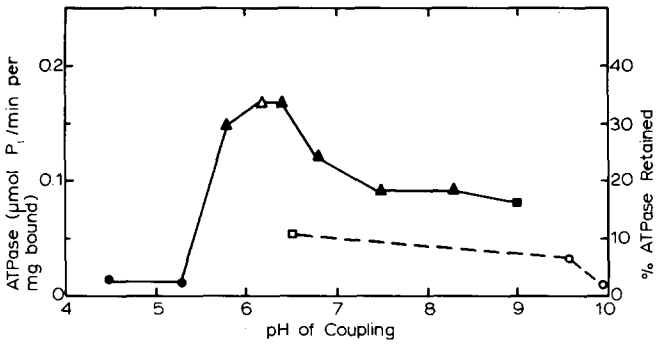


Fig. 1. Retention of EDTA-ATPase as a function of coupling conditions. Myosin was coupled to agarose beads in the presence (solid line) and absence (dotted line) of pyrophosphate at various pH. (Solid symbols: all performed at the same time, open symbols: separate experiments). Coupling reactions were performed in (○) Buffer I, (▲,△) Buffer II, (■) Buffer III, (□) Buffer IV, and (●) Buffer V. ATPase assays were performed under standard conditions and bound protein was determined as described in Materials and Methods.

tions coupled under conditions that optimized the recovery of specific ATPase activity (Buffer II, pH 6.2—6.3).

Determination of coupled protein

The amount of myosin coupled to Sepharose was determined by the amino-acid content of a Sepharose-Myosin hydrolysate. Volumes of beads packed by centrifugation in a graduated tube at 50 × *g* for 5 min were hydrolyzed overnight in constant boiling HCl at 108° C in sealed, evacuated ampules. The quantity of amino acid liberated was determined using fluorescamine [21] and compared to a standard curve of known quantities of myosin hydrolyzed under identical conditions. The values obtained from equal amounts of agarose coupled only with ethanolamine were subtracted from the experimental values to compensate for any primary amino groups that may have arisen from hydrolysis of the CNBr-activated groups. The average correction was less than 1%.

Determination of enzymatic activities

Adenosine triphosphatase assays were performed at 25° C. The standard medium for the EDTA-activated ATPase consisted of 0.5 M KCL, 1 mM EDTA, 2 mM ATP, and 25 mM Tris · HCl, final pH = 8.0. The Ca^{2+}-activated ATPase medium consisted of 50 mM KCl, 4 mM $CaCl_2$, 2 mM ATP and 25 mM Tris · HCl, pH 7.9. The pH dependence of ATPase activity was determined using standard assay conditions except that 0.05 M Tris/histidine/HCl buffer was used. To determine the effects of KCl, Ca^{2+} and ATP on enzyme activity, various concentrations were used as described in the Results. The reaction was initiated by the addition of ATP previously titrated to the reaction pH. Assays of the myosin ATPase contained 0.05—0.20 mg myosin in a total volume of 1 ml and the reaction was terminated by the addition of 0.1 ml of 60% trichloroacetic acid. Assays of the Sepharose-Myosin ATPase contained 0.005—0.050 ml (packed volume) of beads in a total volume of 2 ml. Sepharose-Myosin was dispensed from a stirred suspension using an Eppendorf pipettor with a

widened tip. ATP was added to start the reaction. The tubes were incubated in a tissue-culture-type test-tube rotator to prevent the beads from settling. At the end of the incubation period (5—20 min depending on the amount of material) the reaction mixture was rapidly filtered through a Millipore filter (8 μm pore) to remove the Sepharose-Myosin. Inorganic phosphate was determined by the method of Baginski et al. [22]. A unit of ATPase activity is defined as 1 μmol P_i produced/min. Specific activities were calculated as units/mg for myosin and units/ml packed Sepharose-Myosin for the immobilized complex.

Gel electrophoresis

Electrophoresis in the presence of sodium dodecyl sulfate was performed by the method of Laemmli [23]. Gels were stained with Coomassie Brilliant Blue. After destaining by diffusion, the gels were scanned with a Gilford spectrophotometer equipped with a linear transport. Areas under the peaks were estimated by triangulation.

To determine which polypeptide chains could be released from Sepharose-Myosin under denaturing conditions, aliquots of the derivative were suspended in Laemmli sample buffer [23] containing 1.5% dodecyl sulfate and 5% β-mercaptoethanol, and heated at 100°C for 5 min with frequent agitation. After cooling, the beads were removed by centrifugation, and the supernatants were applied to gels. Protein-bound carbohydrate in the supernatants was determined by the anthrone reaction [24] after precipitation with 8% trichloroacetic acid.

Results

Determination of optimal coupling conditions

The amount of protein bound ranged from 1.8 to 3.5 mg myosin per ml of Sepharose-Myosin, with greater coupling occurring under alkaline binding conditions. The retention of ATPase activity as a function of the coupling pH is summarized in Fig. 1. The activities are expressed as μmol phosphate/min per mg myosin bound, to allow comparison of Sepharose-Myosin samples containing different amounts of protein. The maximum recovery of specific ATPase activity of Sepharose-Myosin is approximately 33% of the ATPase of native myosin. This occurs when the pH of the coupling buffer is pH 6.2—6.4. Repeated preparations coupled at pH 6.3 exhibited similar retention of ATPase. In coupling buffers of Ph $\leqslant$ 5.3 myosin is precipitated. The great loss of ATPase observed in these preparations (2% retention compared with 33% at pH coupling = 6.3) may be a consequence of isoelectric precipitation. A control preparation, in which only ethanolamine was coupled to Sepharose, displayed no ATP hydrolyzing ability.

PP_i is known to interact with myosin in a manner similar to ATP. PP_i can induce an ultraviolet difference spectrum in myosin [25] and can promote the dissociation of actomyosin [26]. PP_i was included in the coupling buffers with the expectation that it would act as an active site protecting agent and help to preserve ATPase activity. Where possible, PP_i was used as the buffering species (pH 5.8—8.3). Outside this range, it was included at a concentration of 10 mM. The recovery of ATPase was far less when PP_i was not present during the

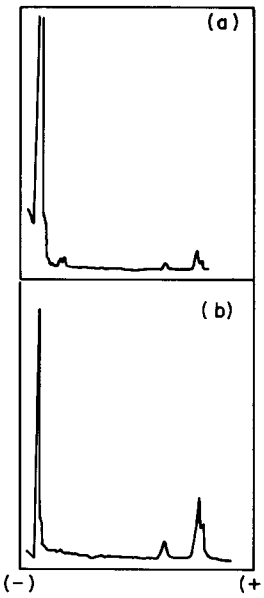
coupling reaction (see dotted line Fig. 1). The use of ATP as a protecting agent was not investigated because ATP is able to form a covalent complex with CNBr-activated agarose [27].

Determination of sites of coupling

Sepharose-Myosin was subjected to denaturing conditions which release noncovalently bound polypeptides and the distribution of released polypeptides was examined by electrophoresis in dodecyl sulfate polyacrylamide gels. Densitometric tracings of myosin and of released polypeptides are shown in Fig. 2. The ratio of heavy chains to light chains (by weight) recovered from Sepharose-Myosin is much lower than that observed in native myosin, indicating that most of the covalent binding of myosin to agarose occurs through the heavy chains. Carbohydrate was not detected in the released polypeptides.

Enzymatic studies

The hydrolysis of ATP by Sepharose-Myosin shows a linear dependence on both time and enzyme concentration in the presence of EDTA (Figs 3 and 4). Similar results were obtained when the Ca^{2+}-activated ATPase was measured. As little as 3 μl (packed volume) of Sepharose-Myosin can be assayed reproducibly without deviation from linearity. Enzymatic activities of several representative preparations are given in Table I. The Ca^{2+}-ATPase of Sepharose-Myosin displays a broad peak in activity at 0.075—0.150 M KCl (Fig. 5).



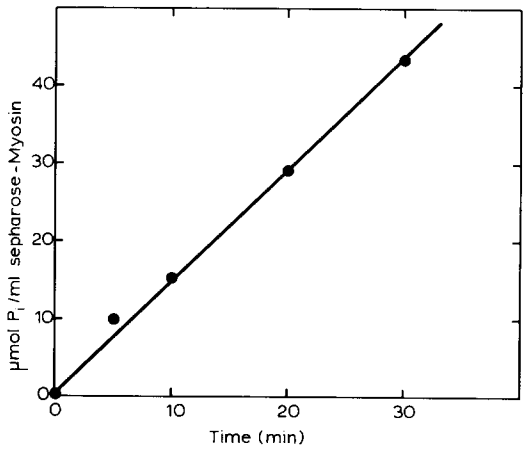


Fig. 2. Densitometric tracings of dodecyl sulfate polyacryamide gels of (a) myosin and (b) polypeptides released from Sepharose-Myosin as described in Materials and Methods. Relative peak areas of (b) are given in Table II.

Fig. 3. Dependence of phosphate production on time. EDTA-ATPase was determined under standard conditions using 0.033 ml Sepharose-Myosin in a total assay volume of 2 ml. ATPase = 1.42 units/ml Sepharose-Myosin. R = 0.998.

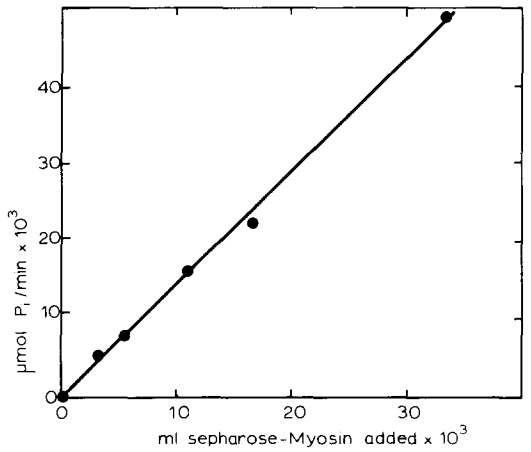


Fig. 4. Dependence of ATPase on enzyme concentration. EDTA-ATPase was assayed under standard conditions with a 15 min incubation. ATPase = 1.45 units/ml Sepharose-Myosin. R = 0.999.

TABLE I

ATPase AND STABILITY OF SOME SEPHAROSE-MYOSIN PREPARATIONS

Preparation	mg myosin bound/ml	ATPase*		Stability at 4°C % of original activity
		EDTA	Ca^{2+}	
Seph-Myo No. 2	3.9	0.25	0.31	12%-7 months[a]
Seph-Myo No. 3	5.8	0.98	—	57%-7 months[b]
Seph-Myo No. 4	—	1.42	1.66	67%-2.5 months[b]
Myosin		0.53	0.45	

* For Sepharose-Myosin, ATPase = μmol P/min per ml Sepharose-Myosin, for myosin, ATPase = μmol P/min per mg.

[a] Stored in unbuffered 0.3 M KCl.

[b] Stored in 0.5 M KCl, 1 mM EDTA, 20 mM Tris · HCl, pH 8.0.

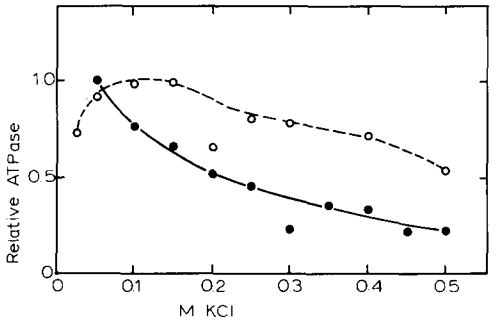


Fig. 5. Effect of KCl concentration on ATPase. The Ca^{2+}-activated ATPase of Sepharose-Myosin (open circles) and myosin (closed circles) were measured under standard conditions with varying concentrations of KCl. Each point represents the mean of 2 experiments, each performed in triplicate. The data are expressed as relative to maximum activity.

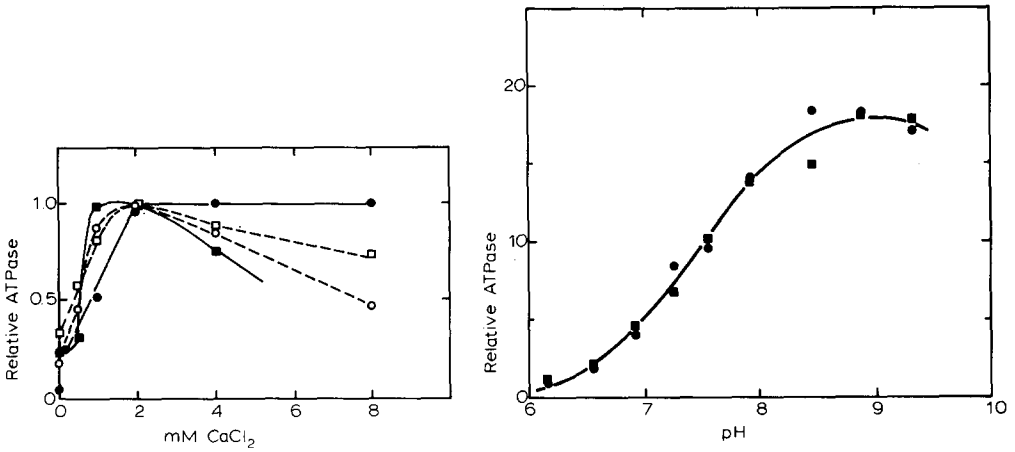


Fig. 6. Effect of $CaCl_2$ on ATPase. Myosin (●,■) and Sepharose-Myosin (○,□) were assayed in 0.05 M (circles) and 0.20 M (squares) KCl.

Fig. 7. Effect of pH on EDTA-ATPase. Assays were performed as described in Methods using 0.02 ml Sepharose-Myosin/2 ml (■) and 0.08 mg Myosin/ml (●). Incubation times were 20 and 10 min respectively. Activities are expressed relative to ATPase at pH 6.0.

At concentrations greater than 0.15 M KCl a decrease in activity is observed. Overall, the sensitivity of the immobilized derivative's ATPase to ionic strength is less than that of free myosin.

Because of the altered response of Sepharose-Myosin to ionic strength, the effect of Ca^{2+} concentration on ATPase was investigated at two KCl concentrations (Fig. 6). At low ionic strength (0.05 M KCl) myosin ATPase shows an asymptotic response to added Ca^{2+} with the plateau in activity occurring at approximately 2 mM $CaCl_2$ (Ca^{2+}/ATP = 1). At the higher KCl concentration (0.2 M), inhibition is observed at concentrations greater than 2 mM $CaCl_2$. The Ca^{2+}-dependence of Sepharose-Myosin ATPase activity differs from that of unbound myosin. At both KCl concentrations (0.05 M and 0.2 M), inhibition is observed at concentrations greater than 2 mM $CaCl_2$.

Displacements of pH-activity profiles of an immobilized enzyme are frequently related to changes in the microenvironment [31]. We examined the pH dependence of the Ca^{2+}- and EDTA-activated ATPase activities to study such effects. The Tris/histidine/HCl buffer system was employed to allow complete experimental control of metal ion concentrations. Fig. 7 shows that the pH dependence of the myosin and Sepharose-Myosin EDTA ATPases are identical with maximal activity occurring at pH 9.0. The effect of pH on the ability of EDTA to chelate trace amounts of inhibitory Mg^{2+} is discussed later. pH-activity profiles for the Ca^{2+}-activated ATPase were performed at three KCl concentrations. The profiles of activity, relative to the ATPase activity at pH 6.0, are shown in Fig. 8. The pH dependence of the Sepharose-Myosin and myosin Ca^{2+} ATPase activities are strikingly similar (Fig. 8). The relative shapes of the pH curves of Sepharose-Myosin follow the same pattern of change with respect to KCl concentration. Specific activities are greater at low ionic strength, in agreement with the results presented in Fig. 5. This relationship is not apparent in

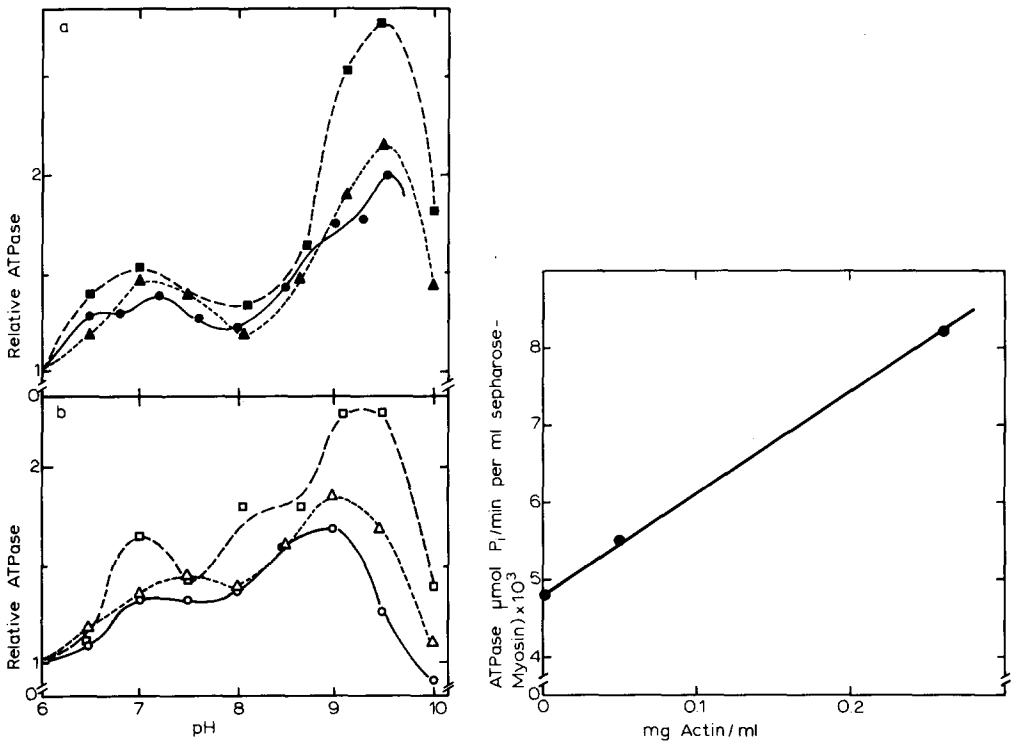


Fig. 8. Effect of pH on Ca^{2+}-ATPase. Activities of myosin (a) and Sepharose-Myosin (b) were determined in 4 mM $CaCl_2$, 2 mM ATP, 50 mM Tris/Histidine/HCl buffer plus 0.05 M KCl (●,○), 0.20 M KCl (▲,△), and 0.50 M KCl (■,□), Activities are expressed relative to ATPase at pH 6.0. Absolute activities decrease with increasing KCl concentration. Note displacement of alkaline pH optimum.

Fig. 9. Actin activation of Sepharose-Myosin ATPase. Assays were performed at 25°C in 5 mM KCl, 2.5 mM $MgCl_2$, 2 mM ATP and 25 mM Tris · HCl pH 7.9 in a total volume of 2 ml. The reaction mixture contained 0.2 ml Sepharose-Myosin and was incubated for 30 min. Each point represents the mean of three determinations. R = 0.999.

Fig. 8 because the data are presented relative to activity obtained at pH 6.0 to allow comparison of pH optima. Both myosin and the immobilized derivative display a characteristic bimodal response to pH. With myosin, optimal activity occurs at pH 7 and pH 9.5 regardless of the ionic strength. Sepharose-Myosin also shows a maximum near pH 7. However, at low and intermediate KCl concentrations the alkaline maximum occurs at pH 9.0, a shift of 0.5 pH units from that of myosin. This pH shift is partially obscured at 0.5 M KCl, where the maximum is broadened and occurs at pH 9.0—9.5.

Many enzymes, when immobilized onto a solid support, are altered in their affinity toward substrate. This effect is noted by an increase or decrease in the apparent Michaelis constant, K'_M [31]. This is also the case with myosin. The effect of varying ATP concentration on the Ca^{2+}- and EDTA-activated ATPase was determined. The steady state K'_M values for Sepharose-Myosin and their associated standard errors, estimated by the weighted statistical method of Wilkinson [32], are summarized in Table III. The parameters obtained for myosin are similar to some reported literature values [6,28]. Upon immobiliza-

tion, the K_M is increased approximately two-fold for both Ca^{2+} and EDTA activities.

In the presence of actin, the Mg^{2+}-ATPase of Sepharose-Myosin is activated or enhanced (Fig. 9). At the highest concentration of actin used, the increase in ATPase activity was 70%. With myosin or heavy meromyosin the ATPase is activated many fold [33,34].

Discussion

Some loss of biological activity usually occurs when a molecule is covalently bound to a surface [10]. Multiple attachments of the protein to the agarose bead is thought to be responsible for this phenomenon [35]. Covalent coupling by the cyanogen bromide procedure occurs through non-protonated primary amino groups [20]. Thus, coupling at any site on the protein molecule is dependent upon the pK of that residue and the pH of the coupling buffer. Higher pH produces more available sites and increases the probability of multiple attachment.

We have shown that myosin can be bound to agarose beads to form a complex with enzymatic activity. The specific ATPase activity of Sepharose-Myosin is highly dependent upon the pH of the coupling reaction and maximum activity is retained when the complex is prepared at pH 6.2—6.4 in the presence of PP_i. Presumably fewer attachments between myosin and the agarose bead occur in this range than at higher pH. Binding in the region of the active site of the myosin molecule is responsible for some of the loss of ATPase activity since the inclusion of PP_i in the coupling reaction increases the retention of ATPase. Perhaps the inclusion of nucleotides (e.g. ITP) that interact with myosin, but are not able to couple to agarose, would further assist in the retention of enzyme activity. The optimum binding pH does not represent the point at which the greatest amount of ATPase is bound, but rather the H^+ concentration that allows the greatest percentage of myosin molecules to retain activity.

The results of the polypeptide-release experiments demonstrate that most of the immobilized myosin is attached through residues located on the heavy chains. As noted above, non-protonated primary amino groups of proteins bind to CNBr-agarose [20]. In myosin, N-terminal amino groups are blocked [36,37] and unavailable for binding. Although very few ϵ-amino groups of lysine would be uncharged at the optimal coupling pH (pH 6.2—6.4), analysis of the relative amounts of heavy and light chains recovered demonstrated a correlation between the binding of any polypeptide and its fraction of total lysine content (see Table II). Since the lysine content of the myosin subunits is approximately constant (86—94 mol/10^5 g for heavy chains and A-1 and DTNB light chains; 67 mol/10^5 g for A-2 light chain) [38], the fraction of total lysine which is contained in any subunit is a function of molecular weight. Thus, for myosin, the probability of each chain binding and the frequency of release is equally related to its molecular weight or lysine content. To demonstrate the dependence of binding probability on lysine content, the binding of other proteins with subunits of greatly varying lysine content would have to be examined.

Assuming that binding is dependent upon the presence of lysine and that

TABLE II

BINDING AND RELEASE OF MYOSIN SUBUNITS OF SEPHAROSE-MYOSIN

Subunit	Mol. wt[a]	lysine[a]	P (Binding)[b]		P (Release)		Relative amount released		
			L	M	(1-L)	(1-M)	Calculated[c]		Observed[d]
							L	M	
Heavy chain[e]	200 000	94	0.86	0.84	0.14	0.16	0.44	0.47	0.50
A_1 (LC 1)	20 000	94	0.04	0.04	0.96	0.96	0.14	0.14	0.14
DTNB[e] (LC 2)	18 500	86	0.07	0.08	0.93	0.92	0.28	0.26	0.26
A_2 (LC 3)	16 500	68	0.03	0.04	0.97	0.96	0.14	0.13	0.10

[a] Molecular weights and lysine contents (mol/10^5 g) of chicken myosin subunits taken from Lowey and Holt [38].

[b] Binding probabilities based on fraction of total lys (L) or weight fraction (M).

[c] Calculations based on lysine fraction (L) or weight fraction (M) and have been normalized. These quantities are an approximation since dye binding to the individual subunits has not been determined.

[d] From densitometric tracing of gel shown in Fig. 2b.

[e] Assuming 2 mol each of heavy chain and of DTNB light chain per mol myosin.

there is no cooperativity between binding sites in the case of multiple attachments, the probability of any chain binding is proportional to the fraction of total lysine present in that polypeptide. The probability of any chain not binding and thus, able to be released by denaturation, is (1-probability of binding) or (1-fraction of total lysine). It follows that the relative amount (by weight) of each polypeptide released is (1-probability of binding) × (weight fraction) or (1-lysine fraction) × (weight fraction). Table II summarizes these calculations. Similar results are obtained whether the calculation of probability of binding is based upon lysine content or the weight fraction. For comparison, the relative amounts of heavy and light chains, determined by densitometry, which have been removed from Sepharose-Myosin are also given in Table II. These values agree well with those predicted on the basis of lysine content or molecular weight. These calculations and data further support our proposal regarding binding sites.

It is unlikely that the release of polypeptides from Sepharose-Myosin is due to the cleavage of agarose. The denaturation was performed near neutrality, whereas the glycosidic bonds of agarose are hydrolyzed only under conditions of extreme pH. Furthermore, cyanogen bromide activation crosslinks the polysaccharide chains [39,40], so that the beads remain intact at high temperature. Carbohydrate was not found in the released polypeptides, but the presence of 1 or 2 sugar residues per myosin molecule may have been beyond the limits of detection.

The enzymatic properties of immobilized myosin are very similar to those of free myosin, yet certain differences exist. These differences include a partial shift of the pH-profile, decreased sensitivity of Sepharose-Myosin to changing KCl concentrations, partial inhibition of ATPase by increasing Ca^{2+} concentrations, and decreased affinity for substrate after immobilization.

These differences between the "bound" and "free" enzyme can be ex-

plained by the relationship of the bound myosin molecules to one another and to the supporting matrix. Close range interaction of myosin molecules is prevented by covalent bindings to the agarose and filament formation cannot occur. The myosin is presumably distributed throughout all parts of the agarose matrix, similar to the distribution that has been demonstrated for IgM, a protein of about twice the molecular weight of myosin [41]. The average concentration of myosin within the Sepharose-Myosin beads is roughly equivalent to the protein concentrations used in laboratory procedures (several mg/ml). The distribution or spectrum of microenvironments of bound myosin molecules (the agarose matrix, other nearby myosin molecules and their associated counterions) differs from that for free molecules in solution. Within the bead there are regions containing substantially higher and lower than average concentrations of enzyme. Further variation in microenvironment results from the fact that the myosin molecules are attached to the matrix through both heavy and light chains. Thus, the overall or averaged enzymatic activities obtained under varying conditions seem to have features of "free" myosin, but still display certain distinct features of "aggregated" myosin due to this spectrum of microenvironments.

Ionic strength-dependent shifts in pH-activity profiles can be caused by charged groups on the supporting matrix [9]. Agarose is a neutral carrier, but the Ca^{2+}-ATPase pH profile of Sepharose-Myosin is displaced 0.5 units toward the acid side. This behavior is independent of ionic strength and is observed only in the alkaline region of the pH curve. If charged acidic groups (e.g. SO_4^{2-}) remaining on the agarose bead were responsible, a shift toward the alkaline side would be seen for both optima and would be abolished by high salt concentrations. Similarly, the removal of titratable lysine residues by the coupling procedure would also result in a shift toward higher pH. We feel that modification of some portion of the myosin molecules at the protein-agarose linkage is probably responsible for the pH shift. This view is consistent with the observation that the pH profile of the EDTA-ATPase is unaltered for Sepharose-Myosin and the sensitivity towards EDTA and Ca^{2+} is reversed. The substrate for the EDTA activity is ATP whereas the $Ca^{2+} \cdot$ ATP chelate is the substrate for the Ca^{2+}-ATPase [42]. Chemical modification may affect one activity and not the other. Limited reaction of myosin with the sulfhydryl reagent *N*-ethylmaleimide also results in altered activation [30].

Upon closer examination, the observed EDTA activated activities may not be due solely to the effect of pH on the enzyme itself, but may be at least partially related to the increased chelating power of EDTA toward trace amounts of Mg^{2+} as the pH becomes more alkaline. At pH 6 the apparent stability constant, K_{app}, for $Mg^{2+} \cdot$ EDTA is $1.12 \cdot 10^4$ and increases to $2.63 \cdot 10^7$ at pH 9. Calculations based upon the data of Offer [43] show that if the total contaminant Mg concentration in our assays was approx. $7.5 \cdot 10^{-6}$ M the activities obtained at pH 7 may be explained completely on the basis of inhibition by Mg^{2+}. This would hold true only if the effect of pH on the enzyme itself was very small compared to the release from Mg^{2+} inhibition. However, if the total trace Mg concentration was slightly lower (10^{-6} M), then inhibition due to free Mg^{2+} would be slight at pH 7 (10—20% inhibition) and insignificant at higher pH. In any case, since the EDTA ATPase pH profiles for Sepharose-

TABLE III

STEADY-STATE MICHAELIS CONSTANTS OF MYOSIN AND SEPHAROSE-MYOSIN ATPase

Preparation	Activator[a]	$K_M \pm$ S.E. (M)[b]	$K'_M \pm$ S.E. (M)[b]
Myosin	Ca^{2+}	$3.31 \pm 0.56 \cdot 10^{-4}$	
Sepharose-Myosin	Ca^{2+}		$6.12 \pm 1.02 \cdot 10^{-4}$
Myosin	EDTA	$1.85 \pm 0.26 \cdot 10^{-4}$	
Sepharose-Myosin	EDTA		$4.32 \pm 0.36 \cdot 10^{-4}$

[a] Ca^{2+}-activated ATPase performed in 0.05 M KCl, 4 mM $CaCl_2$, 25 mM Tris · HCl pH 7.9, and varying concentrations of ATP. EDTA · ATPase: 0.5 M KCl, 1 mM EDTA, 25 mM Tris · HCl pH 8.0, and varying ATP.

[b] Calculated by the method of Wilkinson [32].

Myosin and myosin were identical, we conclude that immobilization has no significant effect on the ion dependency of this activity.

Ionic strength has profound effects on the electrical double layer and hydration of proteins. The effect of ionic strength on myosin aggregation is well known, with myosin monomers favored at high ionic strength [44,45]. Increasing salt concentration also causes inhibition of Ca^{2+}-ATPase activity. The observation that Sepharose-Myosin is not as sensitive to KCl concentration as "free" myosin may be attributed to the prevention of myosin-myosin interactions and to the environment where the hydration provided by the hydrophilic agarose support is fairly constant.

The inhibition of Sepharose-Myosin ATPase by Ca^{2+} may also be a consequence of limited myosin-myosin interactions. Another explanation is provided by the method of preparation. Myosin was coupled to the agarose beads in buffers containing a high concentration of KCl and the immobilization may prevent a return to a low ionic strength conformation.

The apparent decrease in affinity for substrate, as measured by the increase in K_M is in agreement with the investigations on other immobilized enzyme systems [31,45]. Restricted diffusion of the substrate and product within the enzyme beads is thought to be responsible for this effect. The K_M is increased similarly for both the Ca^{2+}- and EDTA-activated activities indicating that if chemical modification takes place, there is no differential effect on substrate affinity.

The activation of the Sepharose-Myosin ATPase by actin was much smaller than that reported for myosin [33] or heavy meromyosin [34]. This can be attributed to several factors. F-actin is a very large molecule and would be excluded from the interior of the beads. Thus, only myosin molecules on the outer surface are available for activation. The restricted mobility of myosin may also play a role, since myosin is fixed to the agarose and cannot saturate the F-actin filament. Increased actin concentrations would be necessary to achieve maximal activation of ATPase activity. The immobilization itself might also alter the optimal conditions of ionic strength, pH, and cation concentration necessary for actin activation.

In summary, we have described procedures for the preparation of an enzymatically active immobilized derivative of myosin that is able to interact,

to some extent, with actin. We have examined the ATPase activity of Sepharose-Myosin, in response to varying ionic conditions. An attempt has been made to understand the effects of immobilization, and prevention of aggregation, on enzymatic activity. We conclude that although Sepharose-Myosin is similar to "free" myosin in several respects, it does exhibit slight modifications at the protein-agarose linkage, retains a fairly constant and stable hydration layer, and does not aggregate or form filaments. In this way we have circumvented the solubility properties of myosin without the use of proteolytic enzymes or harsh chemical treatment. The Sepharose-Myosin complex appears useful as a system for the further study of muscle and non-muscle myosins and their interaction with other macromolecules participating in motile activity.

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