Applications of Immobilized Adenosine Triphosphate in the Study of Myosin†

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ABSTRACT: Agarose-bound adenosine triphosphate (ATP) columns have been prepared which are capable of specifically binding active myosin and one-headed myosin at a high ionic strength as well as active heavy meromyosin (HMM) and HMM subfragment 1 (HMM S-1) at both high and low ionic strengths. The double-headed myosin and HMM were found to have a greater affinity for a column than one-headed myosin and HMM S-1, respectively. Removal of inactive material and preparation of fully active and more homogeneous species could thus be achieved. The binding of myosin and one-headed myosin required the presence of Ca^{2+} or Mg^{2+} ions in the millimolar range and was accompanied by the splitting of the bound ATP. Thus, binding in this case is not a reversible process and is intimately associated with the occurrence of a chemical process. One is therefore faced with a new type of affinity chromatography for which we suggest the term "dynamic affinity chromatography." Bound ATP columns are currently used in our laboratory as a routine procedure for the preparation of purified active myosin species.

Recently we have reported the preparation of Sepharose-ATP which could specifically interact with heavy meromyosin (HMM)[1] (Lamed *et al.*, 1973); moreover, indirect evidence suggested that only active HMM, *i.e.*, HMM exhibiting ATPase activity, was adsorbed. The use of columns filled with insolubilized NTPs for the separation of myosin and of its active subfragments was suggested and discussed. In the following we shall describe various applications of such ATP affinity chromatography columns. These include the separation between active and thermally (or chemically) inactivated HMM (or HMM subfragment-1, HMM S-1) and the partial separation between the double-headed HMM and the one-headed HMM S-1. As the previously described Sepharose-ATP did not adsorb myosin at a high ionic strength, a different ATP column was prepared which was capable of adsorbing myosin, as well as one-headed myosin, provided divalent ions were present. Our studies led to the development of routine procedures for the preparation of pure myosin fragments, utilizing ATP columns.

Materials and Methods

Chemicals. Trypsin was obtained from Worthington Biochemical Corp. and papain from Miles Yeda, Rehovot, Israel. Agarose (Sepharose 2B and 4B) was produced by Pharmacia and ATP was a Sigma chemical.

[γ-^{32}P]ATP was prepared according to Avron (1960).

Preparation of Sepharose Hydrazides and Coupling of ATP. Sepharose 2B-adipic acid hydrazide-ATP (Seph-ADH-ATP) was prepared following the procedure we described earlier (Lamed *et al.*, 1973).

Sepharose 4B-sebacic acid hydrazide-ATP (Seph-SEH-ATP) was prepared as follows. Cyanogen bromide activated Sepharose 4B was suspended in two volumes of sebacic acid dihydrazide solution (prepared similarly to adipic acid dihydrazide) in acetic acid at 20° (20 g/l.). The reaction was allowed to proceed with stirring for 16 hr at 20° and the product was washed with 0.06 N HCl and water. Coupling of periodate-oxidized ATP to this was carried out as in the preparation of Seph-ADH-ATP. The bound ATP content was 3–4 μmol/g of wet gel.

Preparation of Myosin. This was performed according to Azuma and Watanabe (1965) using rabbit's white back muscle or chicken white breast muscle (to which we shall refer thereafter as rabbit and chicken myosin, respectively).

Preparation of HMM. Rabbit or chicken HMM was prepared following the procedure published by Tokuyama *et al.* (1969). In some cases, crude HMM, *i.e.*, prior to the ammonium sulfate fractionation, was used while in others it was affinity chromatographed prior to its use (see section c of the Results).

Preparation of HMM S-1. The methods described by Lowey *et al.* (1969) utilizing rabbit or chicken myosin were followed. The myosin was digested either while suspended in 0.2 M ammonium acetate by papain, or in solution in 0.5 M KCl by insoluble papain. "Crude" HMM S-1, prior to DEAE-cellulose chromatography, was used in several experiments and in others it was purified by a Sepharose-ATP column (see section c of the Results).

Preparation of Crude One-Headed Myosin. For the digestion of myosin, the procedure utilized was essentially that of Margossian and Lowey (1973).

A suspension of fresh chicken myosin in 0.2 M ammonium acetate (pH 7.2) was prepared (10 mg/ml). Papain which was preactivated (in 0.005 M cysteine-0.002 M EDTA) was then added to give a final concentration of 8 μg/ml. The digestion was allowed to proceed with stirring for 6 min at 25° and was stopped by the addition of iodoacetic acid to a concentration of 1 mM. The precipitate was collected by centrifugation at 17,000 rpm for 20 min, washed by suspension in water, centrifuged, and dissolved in 0.5 M KCl.

ATPase Assays. These were carried out at 25°.

a. Ca^{2+}-ATPase. The reaction mixture (3 ml) was 0.5 M in KCl, 4 mM in $CaCl_2$, and 2.5 mM in ATP and contained adequate amounts of myosin, HMM, or HMM S-1 to be determined. The reaction was followed in a pH-Stat at pH 7.5 with 0.01 or 0.02 N NaOH as titrant.

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[1] Abbreviations used are: HMM, heavy meromyosin; LMM, light meromyosin; Seph-ADH-ATP, Sepharose 2B-adipic acid hydrazide-ATP; Seph-SEH-ATP, Sepharose 4B-sebacic acid-ATP.

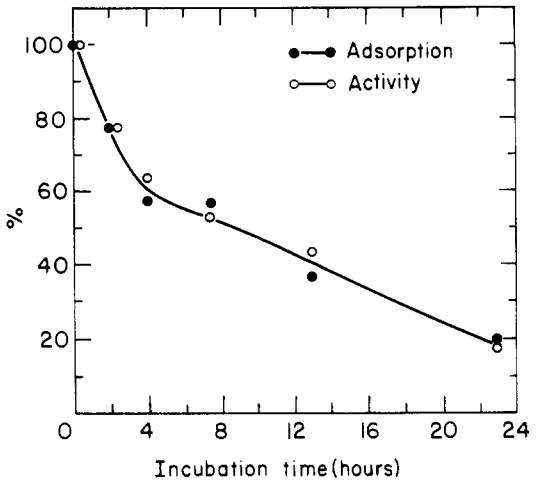

FIGURE 1: Thermal inactivation of HMM S-1. A solution of purified rabbit HMM S-1 (2 mg/ml), 50 mM KCl, 10 mM imidazole buffer (pH 7), 0.2 mM EDTA, and a drop of toluene to prevent bacterial growth was incubated at 25°. Aliquots were withdrawn after the indicated periods of time and Ca^{2+}-ATPase was measured; 0.5 ml of treated HMM S-1 was added to a suspension of 1 g of wet Seph-ADH-ATP in 2.5 ml containing also 25 mM KCl, 10 mM imidazole buffer (pH 7), and 1 mM EDTA. After 15-min stirring in the cold the suspension was centrifuged and the protein in the supernatant was determined. The absence of remaining Ca^{2+}-ATPase activity was ascertained. The percentage of adsorbed protein was calculated using the value at zero time as 100% (85% of the total protein).

b. EDTA-KCl ATPase. The assay conditions were similar to (a) except that $CaCl_2$ was replaced by EDTA (2 mM).

SODIUM DODECYL SULFATE-GEL ELECTROPHORESIS. This was performed essentially as described by Weber and Osborn (1969) using 5% polyacrylamide gels.

Protein determination was carried out by the Lowry procedure (Lowry *et al.*, 1951).

Results

a. Inactivation Experiments. Figure 1 shows very clearly that the adsorbability of HMM S-1 kept at 25° to Sepharose-adipic hydrazide-ATP decreases in parallel to the fall in Ca^{2+}-ATPase (or in EDTA-KCl activation). In other words, it appears that only active heads can bind to the column. The proportionality between ATPase activity and amount of bound protein was kept even though the exact shape of the inactivation time curve could vary from one experiment to another. For heat-denatured HMM, the fall in ATPase activity with time was accompanied by a decrease in the amount of adsorbed protein (Figure 2). In this case, contrary to the HMM S-1 case, loss of adsorbability was not proportional to the loss in ATPase activity; the per cent decrease in adsorbability was smaller than the corresponding decrease in ATPase as long as the myosin was not fully inactivated. The same result was obtained when EDTA-KCl was measured instead of Ca^{2+} activation. This difference in the behavior of HMM and of HMM S-1 is to be expected if one assumes that HMM will bind to the column even if it is left with one active head only.

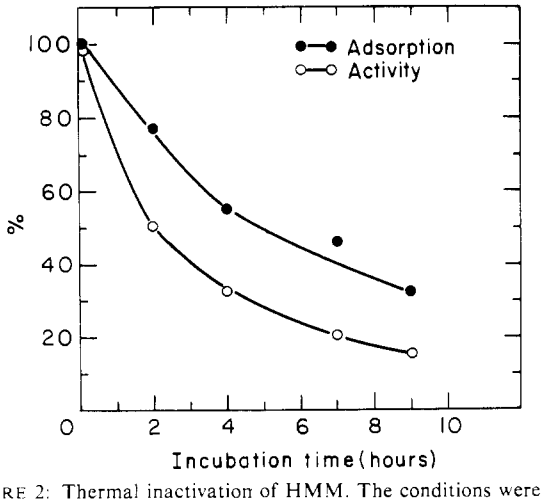

FIGURE 2: Thermal inactivation of HMM. The conditions were similar to those described in the Legend to Figure 1, using purified rabbit HMM.

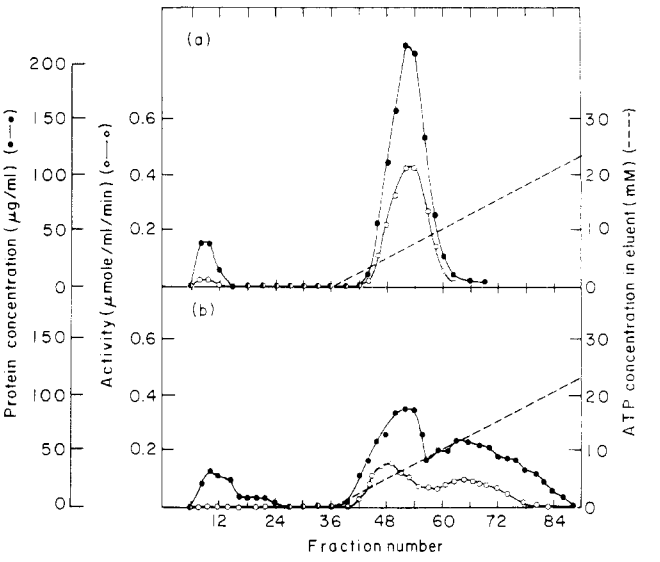

FIGURE 3: Affinity chromatography of HMM S-1 and of mixtures of HMM and HMM S-1. 2.7 mg of purified chicken HMM S-1 (a) or 1.5 mg of chicken HMM S-1 plus 2.5 mg of chicken HMM (b) in 5 ml of 10 mM Tris-HCl buffer (pH 7.6) and 2 mM EDTA were loaded on a 1.1 × 10 cm column of Seph-ADH-ATP (1 μmol of bound ATP/ml of wet wt gel). Fractions (1.3 ml) were collected from each column by first washing with 10 mM Tris-HCl buffer (pH 7.6), containing 2 mM EDTA and then eluting with a linear gradient of ATP dissolved in the same buffer solution. Ca^{2+}-ATPase of the fractions was determined as described in Materials and Methods except that the KCl concentration was 0.05 M. The increase in ATP concentration of the assay solution due to ATP in the eluent did not exceed 0.7 mM.

Inactivation was also brought about by a short treatment of HMM with alkali at pH 11. This is known to release the "alkali light chains" of myosin. The treatment caused complete abolishment of both ATPase activity and adsorbability by the Sepharose-adipic dihydrazide-ATP column. The same results were obtained with myosin fully inactivated by *p*-chloromercuribenzoate.

b. Separation of Mixtures of HMM and HMM S-1 by Affinity Chromatography. It was plausible to assume that if the density of available bound ATP groups is sufficiently high, the affinity of binding of the double-headed molecules to a given column should be higher than that of the corresponding single-headed species. In Figure 3a we present the affinity chromatography of purified HMM S-1 (Lowey *et al.*, 1969), using an ATP gradient for elution. As can be seen from the figure, most, but not all, of the preparation appears as a single symmetrical and relatively sharp peak. HMM prepared according to Tokuyama *et al.* (1969) from both chicken (white breast) and rabbit (back) muscle gave a somewhat broader peak under similar conditions. In Figure 3b we show the partial resolution of a mixture of HMM and HMM S-1 into two peaks. Comparison of Figure 3a,b shows that, as expected, HMM S-1 is less strongly bound to the column than the double-headed HMM. The use of KCl gradients instead of ATP gradients proved to

be less effective as regards resolution. Similar columns containing a higher content of bound ATP required much higher concentrations of ATP for elution while the KCl concentrations needed were not markedly affected. It is quite probable that the resolving power of the ATP columns (with respect to mixture of one- and two-headed species) will increase with increasing bound ATP content since at relatively low concentrations both types will interact *via* one head only.

c. Use of Seph–ADH–ATP Columns for the Purification of HMM and HMM S-1. The results described above led us to the routine use of Sepharose–ATP columns for the preparation of purified active HMM and HMM S-1. Continuous use of these columns is evidently possible since, as we have shown in the previous publication, the bound ATP is not split by the bound myosin fragments in the absence of Ca^{2+} and Mg^{2+}. Figure 4a describes the purification of crude HMM S-1. This was prepared by papain digestion of rabbit myosin, suspended in 0.2 M ammonium acetate. The myosin rods were removed by centrifugation after dilution or dialysis against a low ionic strength solution. The crude HMM S-1 was adsorbed by the columns and after washing was eluted in one step by 0.7 M KCl. The first peak exhibited a low fraction of the total ATPase activity even though it contained about 40% of the protein.

The specific activity of the HMM S-1 eluted from the second peak was about twice as high as that of the original preparation. It should be noted that, at the same time, the gel electrophoretic pattern of the pure HMM S-1 did not differ much from that of the crude preparation. This obviously reflects the fact that while sodium dodecyl sulfate–gel electrophoresis cannot distinguish between originally active and inactive species of the same molecular weight, our method as presented in section a of Results is capable of differentiating between active and inactive HMM S-1 (as well as HMM). In other similar experiments the sodium dodecyl sulfate–gel electrophoretic pattern of the crude HMM S-1 was more complex than in the experiment described in Figure 4a but, at the same time, the pattern of the purified material was identical with that presented in the figure. Since the faster, minor, band appeared in all experiments, this appears to correspond to a digestion product of HMM S-1 which separates by the action of sodium dodecyl sulfate (it is too heavy to be considered as one of its light chains). It is worth mentioning that purified HMM S-1 prepared from chicken myosin exhibited practically the same sodium dodecyl sulfate–gel electrophoretic pattern.

Figure 4b represents the behavior of HMM. The HMM was prepared by tryptic digestion of rabbit myosin in 0.5 M KCl, dialysis against a low ionic strength solution, and removal of light meromyosin (LMM) by centrifugation. Elution was again carried out in one step. The pattern of elution is quite similar to that of HMM S-1, practically all the ATPase activity residing in the second sharp peak which evidently has a higher specific activity than the original preparation. The main difference between the sodium dodecyl sulfate–gel electrophoretic run of the crude and the purified HMM is the disappearance of the heaviest components (which might be myosin) in the latter. Ammonium sulfate fractional precipitation of the crude HMM, which is commonly used for the purification of HMM, did not help in removing these bands or in significantly increasing the specific activity, nor did high-speed centrifugation for the removal of LMM prove to be helpful in these respects.

Recycling through the column of the purified HMM or HMM S-1 after dialysis or $(NH_4)_2SO_4$ precipitation and dialysis gives practically one single peak. When HMM prepared according to Tokuyama *et al.* (1969) was treated with a Seph–ADH–ATP column immediately after $(NH_4)_2SO_4$ fractionation, about 15% of the protein was not adsorbed and did not exhibit any ATPase activity. Upon eluting with a KCl gradient, a single, active, peak was observed. Similarly, all HMM S-1 preparations obtained according to Lowey *et al.* (1969) exhibited a peak of inactive material whether they were or were not previously chromatographed by DEAE-cellulose. The specific activity of the major, second, peak was higher by about 10% than that of the original preparation after DEAE-cellulose treatment.

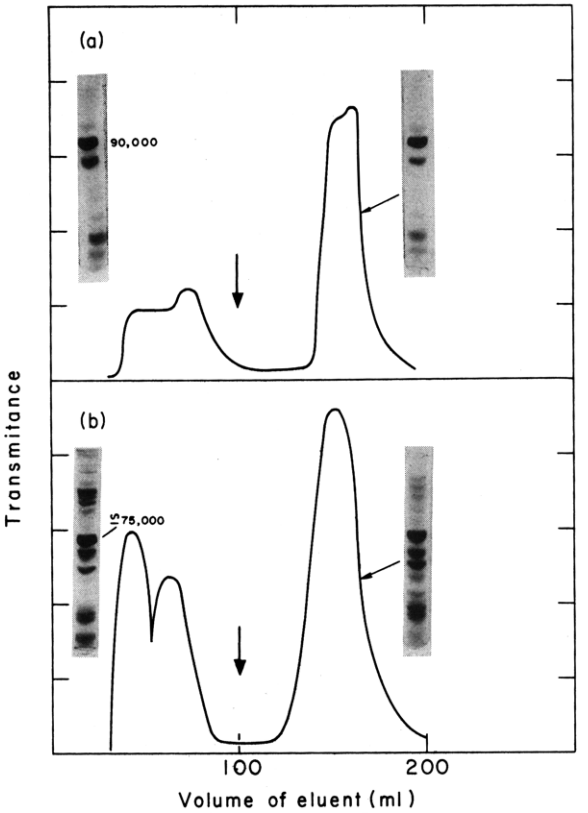

FIGURE 4: Preparative affinity chromatography of HMM S-1 and HMM. A 2 × 17 cm column of Seph–ADH–ATP, previously washed with a 40 mM KCl, 10 mM Tris-HCl buffer (pH 7.6), and 1 mM EDTA solution was loaded with (a) 120 crude rabbit HMM S-1 or (b) 200 mg of crude rabbit HMM in 40 ml of the equilibrating solution. Elution at a flow rate of 200 ml/hr was performed by first washing with 100 ml of the equilibrating solution, followed by 100 ml of a 0.7 M KCl, 10 mM Tris-HCl (pH 7.6), and 1 mM EDTA solution, in order to desorb the protein. The ultraviolet transmittance was automatically recorded in order to trace the protein elution. Sodium dodecyl sulfate–gel electrophoresis patterns of the combined second peaks and of the initial crude preparations are shown in the figure.

In a typical experiment, myosin with a Ca^{2+}-ATPase activity of 0.7 μmol/mg per min served for the preparation of crude HMM (*i.e.*, without $(NH_4)_2SO_4$ fractionation). The activity of the latter was 0.8 μmol/mg per min and became 1.5–1.6 μmol/mg per min after purification with a Seph–ADH–ATP column. Crude HMM S-1 was prepared from the same myosin, suspended in 0.2 M ammonium acetate by using soluble papain for digestion (Lowey *et al.*, 1969). The material was purified by a Seph–ADH–ATP column instead of DEAE-cellulose, giving a specific activity of 2.1–2.2 μmol/mg per min. The activity of HMM S-1 prepared from the same myosin in 0.5 M KCl with insoluble papain and purified by DEAE-cellulose (Lowey *et al.*, 1969) was 1.8 μmol/mg per min at the center of the DEAE-cellulose peak. The ATPase activities given above were determined at 25° in the presence of 50 mM KCl, 4 mM $CaCl_2$, and 2.5 mM ATP at pH 7.6.

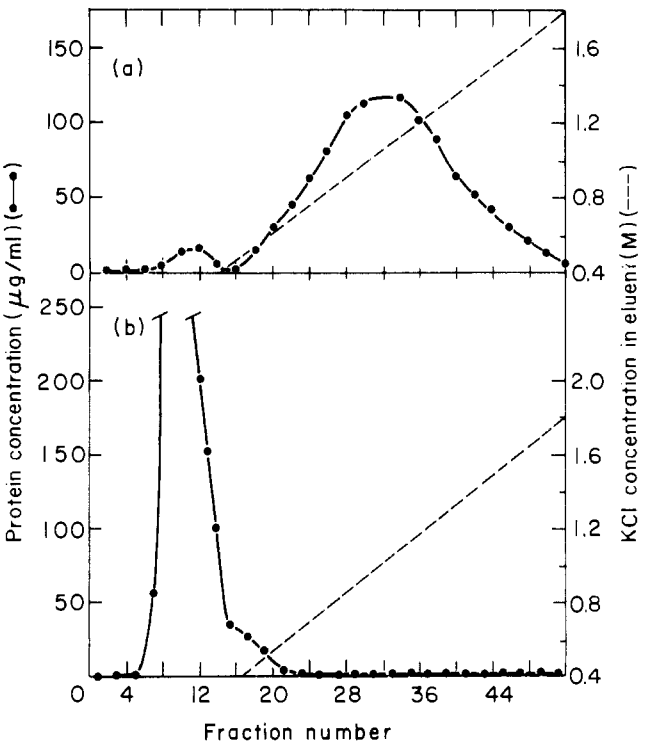

FIGURE 5: Column chromatography of chicken myosin using Seph-SEH-ATP. Myosin (6 mg) in 2 ml of a 0.4 M KCl-10 mM borate buffer (pH 8.2) was poured over a 1.1 × 15 cm column of Seph-SEH-ATP, previously washed by a solution containing: (1) 0.4 M KCl, 10 mM borate (pH 8.2), 1 mM EDTA, and 3 mM $CaCl_2$; (b) the same without $CaCl_2$. Fractions (2 ml) were collected from each column by first washing with the equilibrating solution and then eluting with a linear KCl gradient.

In the following some data which are relevant to the practical utilization of the Seph-ADH-ATP columns are given.

In the presence of EDTA, a column retained practically all its original capacity (about 2 mg of purified protein/ml wet gel) after cycling HMM and/or HMM S-1 eight times within 3 weeks.

The protein concentration pooled from the center of an active peak (60–70% of the total peak protein) is usually about 4 mg/ml while that of the rest of the peak is 1–2 mg/ml. These concentrations are suitable for many purposes without requiring further concentration.

d. Affinity Chromatography of Myosin. While Seph-ADH-ATP was found to be capable of binding HMM and HMM S-1 at low ionic strength, our previous efforts to adsorb significant amounts of myosin under the high ionic strength conditions needed for its disaggregation were unsuccessful (Lamed *et al.*, 1973). Increasing the content of bound ATP up to 7–8 μmol/ml of Sepharose did not prove to be helpful. The properties of the gels were not affected by reduction of the bond between ATP and the hydrazide group with sodium borohydride. We have therefore attempted to bind the ATP to the Sepharose *via* a longer extension arm which in many other cases proved to be effective. The six-carbon adipic dihydrazide was substituted by the ten-carbon sebacic dihydrazide. Since it was previously observed (Lamed *et al.*, 1973) that the adsorption of HMM by Sepharose-adipic hydrazide-ATP could be influenced by Ca^{2+} and Mg^{2+} ions, we tested the retention of myosin by Seph-SEH-ATP columns both in the presence and in the absence of these divalent cations. As can be seen from a comparison of parts a and b of Figure 5, practically full retention was found in the presence of Ca^{2+} ions (similar results were obtained in the presence of an equal concentration of Mg^{2+}) while in their absence the interaction was much weaker.

As previously reported by us, both Ca^{2+} and Mg^{2+} ions activate the splitting of bound ATP in Seph-ADH-ATP by HMM. The release of P_i was therefore followed under conditions similar to those prevailing in the experiments shown in Figure 5a,b (using rabbit myosin). For this purpose, [γ-^{32}P]Seph-SEH-ATP was utilized; 6.7% of the original ATP bound was decomposed. Upon using the same column for a second time, an additional 4.5% of the original ATP was split. The binding capacity of the column (5 g wet gel) was smaller (1.2 mg) in the second run in comparison with the first run (1.5 mg). A third run caused the release of 2.9% of the original radioactivity; the amount of tightly bound myosin was now only 0.95 mg, *i.e.*, only 64% of the capacity in the first run. There were cases in which myosin could not be absorbed after the column was used only once or twice. In some experiments we found that myosin absorbed by the column could be removed by the equilibrating buffer itself (0.4 M KCl–10 mM borate (pH 8.2)–1 mM EDTA–3 mM Ca^{2+}) if we let the myosin stay in the column for several hours (Figure 5). Similarly, HMM S-1 which was tightly absorbed on a Seph-ADH-ATP column in the presence of Ca^{2+} at *room temperature* could be eluted in active state by washing with a low ionic strength solution containing 10 mM Tris-HCl buffer (pH 7.6) and 2.5 mM Ca^{2+}; protein, ATPase activity, and phosphate determinations each gave a single peak, the phosphate peak somewhat preceding that of the protein. In these two cases, the enzyme probably has an opportunity to fully exhaust the ATP (as a result of a long stay in the case of myosin and because of the relatively high temperature in the case of HMM S-1). The protein could thus come off the column without raising ionic strength or adding ATP. We also observed that a higher KCl concentration (about 0.5 M) was required in order to elute HMM from a Seph-ADH-ATP column in the presence of Ca^{2+} than in the presence of EDTA (about 0.25 M KCl). In another experiment, HMM S-1 was adsorbed by a Seph-SEH-ATP column at a low ionic strength. When Ca^{2+} was present, elution required about the same KCl concentration as for myosin, *i.e.*, above 0.5 M KCl while in the presence of EDTA about 0.2 M KCl was sufficient for elution.

On the other hand, a Seph-SEH-ATP column operating under such conditions that no myosin was adsorbed (*i.e.*, in the absence of Ca^{2+}) did not release any phosphate; moreover, such an operation did not alter the binding capacity of the column.

Elution patterns similar to those shown in Figure 5 have been obtained with ATP as eluent. It should be noted that the major band in Figure 5a is quite broad; in many similar experiments the peak was less symmetrical. This might be ascribed either to the fact that the myosin molecule is highly asymmetrical and steric factors might play a role in its adsorption and elution or, alternatively, to an heterogeneity of the myosin molecules due to the coexistence of several isoenzymes. Most of the protein and of the original ATPase activity were recovered by the time elution was completed. The operation of steric factors is suggested also by the fact that the binding capacity of Seph-SEH-ATP columns for myosin appears to drop more rapidly than the bound ATP content, as indicated by phosphate release. Preliminary experiments indicate that the smaller and less asymmetrical HMM S-1 binds to a larger extent than myosin to this column under the same conditions.

Utilizing Seph-SEH-ATP columns, we tried to isolate one-headed myosin from a myosin preparation that had been treated with papain for a short time. The complete removal of

HMM S-1 after precipitation at a low ionic strength was evident from a comparison of sodium dodecyl sulfate–gel electrophoretic patterns of the original and the precipitated preparations. The electrophoresis of the latter exhibited two major bands, one corresponding to the heavy chain of myosin (about 200,000 daltons) and a faster moving band of 110,000–120,000 corresponding to a myosin heavy chain which has lost its S-1 end (Figure 6). The protein elution pattern showed, in addition to the small inactive peak obtained before the KCl gradient was applied (and which might contain also myosin rods), two close peaks, while that of the ATPase exhibited a single asymmetrical peak with a maximum at a location corresponding to the second peak of the protein. Sodium dodecyl sulfate–gel electrophoresis of pooled fractions 36–40 (the first peak) gave two bands corresponding to a 120,000- and a 200,000-dalton species. The second protein peak (fractions 51–54) gave mainly the 200,000-dalton band. It thus appears that the second peak contains mainly intact myosin (each molecule of which is known to contain two heavy chains of 200,000 daltons each) while the first peak contains mostly one-headed myosin which is split by sodium dodecyl sulfate into one intact and one chopped heavy chain. Further support for this conclusion may be derived from the fact that the specific ATPase activity is higher in the second peak than in the first one. This can be attributed to the fact that the unit molecular weight per active site of myosin is 250,000, while one-headed myosin has a molecular weight of about 380,000. Assuming that the protein at the extreme left side of the peak is pure one-headed myosin while that at the right side is pure myosin, we calculated the amount of each of these substances at each point. The first peak was found to correspond almost entirely to one-headed myosin. Fractions 51–54 appeared to contain mainly myosin, in accordance with the gel pattern. The two-headed species has, as in the case of a mixture of HMM and HMM S-1 (Figure 3b), a higher affinity for the ATP column than its one-headed analog. We are currently investigating the use of ATP and pyrophosphate, rather than KCl gradients, as well as the replacement of Ca^{2+} by Mg^{2+}, in order to attain a better resolution in this system.

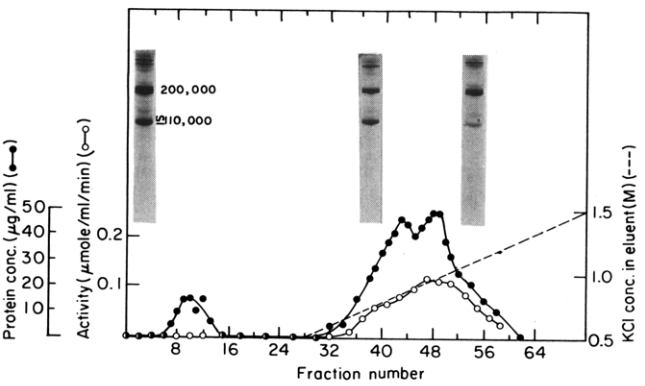

FIGURE 6: Affinity chromatography of crude one-headed myosin (containing also intact myosin). Crude chicken one-headed myosin (3.5 mg) in 1 ml of 0.5 M KCl was poured over a 1.1 × 12 cm column of Seph-SEH-ATP previously washed by a solution containing 0.5 M KCl, 10 mM Tris-HCl buffer (pH 7), and 4 mM $CaCl_2$. Fractions (2 ml) were collected by first eluting with equilibrating solution and then using a linear KCl gradient. EDTA-KCl, ATPase, and protein were measured. Fractions 36–40 and 51–54 of the first and second peaks were pooled, dialyzed against water, lyophilized, and the products analyzed by sodium dodecyl sulfate–gel electrophoresis. The patterns, including that of the initial mixture, are shown in the figure.

Discussion

Our results support the belief we expressed earlier that immobilized ATP and other nucleotides may be used in affinity chromatography columns for the purification of active myosin and myosin derivatives. In particular, ATP columns appear to be capable of distinguishing between active molecules of a given myosin species and accompanying inactive ones. The removal of the latter should prove to be useful especially in studies of the stoichiometry and energetics of binding of the myosin species to actin, ATP, etc.

A comparison of the different relationships between adsorbability and ATPase activity exhibited by HMM and by HMM S-1 (see Figures 1 and 2) implies that both heads in HMM are capable of being adsorbed to bound ATP.

The fact that myosin and HMM have two heads, each of them being capable of forming a complex with bound ATP and splitting it in the presence of divalent ions enabled us to differentiate between species containing one and two active heads, respectively. The difference in affinity arises in the first place from the greater statistical probability of a two-headed molecule binding to a given ATP site and from the lesser probability of its fully dissociating when both its active heads are bound to ATP. In addition, the proximity of the two heads should increase by a large factor the chance of the second head binding once the first has been bound. Obviously, cooperative and anticooperative effects might be superimposed on the purely statistical factors, and these could either increase or decrease the ratio of the affinities of the two- and one-headed molecules.

The observation that myosin or its active subfragments at high ionic strength bind to Seph-SEH-ATP only under conditions which lead to the splitting of the bound ATP, and the fact that they are not absorbed under the same conditions by columns to which we have bound ADP or AMP-PNP (adenylyl imidodiphosphate (Yount *et al.*, 1971)), neither of which can be split by myosin (Muhlrad, Lamed, and Oplatka, in preparation) suggests that it is the intermediary products of the hydrolysis reaction of the ATP which are responsible for the adsorption.

Recently we have found (Muhlrad, Lamed, and Oplatka, in preparation) that, in the presence of magnesium ions trinitrobenzenesulfonate-treated myosin splits ATP in Seph-SEH-ATP, even though at a lower rate than native myosin. In parallel, the binding of trinitrobenzenesulfonate–myosin or of its active subfragments to the column in the presence of Mg^{2+} is stronger, and higher KCl concentrations are required for their elution than for their unmodified analogs. Thus, two discrete peaks were obtained upon eluting a mixture of myosin and trinitrobenzenesulfonate–myosin by a KCl gradient. On the other hand, in the presence of EDTA, a mixture of these proteins gave a *single* peak (both were washed out together without applying an eluent). The difference in affinity of binding to the column in the presence of Mg^{2+} may thus be associated with difference in ATPase activity. We are at present investigating the possibility of separating myosins of slow and fast muscles by using ATP columns.

Affinity chromatography generally involves the complexing of a large molecule with a ligand bound to an insoluble matrix. The interaction is generally reversible and the complex can be dissociated with a proper eluent. Thus, each ligand unit may undergo many association–dissociation cycles. ATP columns, operating under conditions in which the ATP ligand is split, may present us with a new type of affinity chromatography columns in which the affinity of binding is determined by the course of an irreversible chemical reaction between the ligand and an enzyme. We suggest the term "dynamic affinity chromatography" for this type of process. Columns operating on

this basis may prove useful in the investigation of native and of chemically modified active sites of enzymes.

The fact that the elution pattern of purified HMM S-1 in the presence of Ca^{2+} exhibits a single, symmetrical, and quite sharp peak suggests that both myosin heads can split bound ATP, apparently with the same activity. This can also account for the stronger affinity of binding of myosin in comparison to one-headed myosin under conditions in which ATP is split (as can be seen from Figure 6).

Acknowledgment

We thank Dr. Andras Muhlrad for his help and advice.

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Chemical Polymerization of Oligonucleotides Directed by a Complementary Polynucleotide. Preparation and Polymerization of Oligo(2′-*O*-methylinosine 3′-phosphate)†

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ABSTRACT: Substantial quantities of oligo(2′-*O*-methylinosinates) with 3′-terminal phosphates and defined chain lengths (n = 2–12) have been prepared by controlled hydrolysis of poly(2′-*O*-methylinosinate) with micrococcal nuclease, followed by DEAE-cellulose column chromatography. Two oligonucleotide fractions, hexa(2′-*O*-methylinosine 3′-phosphate) and penta(2′-*O*-methylinosine 3′-phosphate), have been used as starting materials in a polymerization reaction directed by a poly(C) template. These reactions were carried out in aqueous solution at low temperature (0 or −15°) with a water-soluble carbodiimide as the activating agent. The absolute overall yield was 38–61%, and the relative overall yield based on the recovered material was 43–71%; the yield of the 30-mer fraction (product with 5–6 linkages) can be as large as 15%. The stability of the 1:1 oligo(2′-*O*-methylinosine)·poly(C) complex is an important factor in determining the extent of the polymerization and the chain length of the product.

Synthesis of long-chain polynucleotides of defined sequence in sufficient quantity has been a very formidable challenge. Development of synthetic chemistry in the past decade has provided the methodology for the synthesis of oligonucleotides (especially deoxyribosyl oligomers) with a chain length of less than ten (10-mer or smaller) in reasonable yield in return for the effort and the cost. One logical approach to the synthesis of longer polynucleotides (n = 50–100) is to condense the preformed oligomers (n = 5–10). An early attempt using this strategy of block condensation by the conventional method under anhydrous conditions was not promising (*e.g.*, see Jacob *et al.*, 1967). An alternative approach is to use a complementary polynucleotide as a template to concentrate the appropriate oligomer substrates into a small area with proper alignment. Such a reaction has to be carried out in aqueous solution usually with a water-soluble carbodiimide as the activating agent. In 1966, Naylor and Gilham reported that $d(pT)_6$ was condensed in the presence of a poly(A) template to afford mainly $d(pT)_{12}$ in 5% yield. Orgel and coworkers, with their interest principally in the mechanism of prebiotic synthesis of nucleic acid, have investigated the condensation of adenine nucleotides in the presence of complementary polyuridylate (Sulston *et al.*, 1968, 1969; Schneider-Bernloehr *et al.*, 1968; Weimann *et al.*, 1968; Renz *et al.*, 1971). In this series of studies, water-soluble carbodiimide was first used as an activating agent for the 5′-nucleotides; subsequently the preactivated nucleotides, such as imidazolides of adenosine 5′-monophosphates, and adenosine 2′,3′-cyclic phosphates were used. In 1970, Shabarova and Prokofiev reported the polymerization of the amino acid amidate of $d(pA)_2$ in the presence of a poly(U) template which gave a 10% yield of the polymerized products. Another alternative for the template-directed oligonucleotide polymerization recently developed is the use of the joining enzyme, the polynucleotide ligase. The successful use of this enzymic method not only allowed the joining of deoxyribosyloligonucleotides (Gupta *et al.*, 1968; Harvey and Wright, 1972) and ribosyl oligonucleotides (Kleppe *et al.*, 1970), but also the synthesis of

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