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ENZYMATIC MECHANOCHEMISTRY

I. THE INTERACTION OF HEAVY MEROMYOSIN WITH "IMMOBILIZED ADENOSINE TRIPHOSPHATE"

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SUMMARY

ATP was covalently bound to an agarose gel. The insolubilized ATP was found to be capable of specifically binding heavy meromyosin. The adsorbed heavy meromyosin could be eluted by ATP in solution. Both binding and elution by ATP of heavy meromyosin were not much effected by Ca^{2+}, Mg^{2+} or EDTA.

While the water-soluble polyalanine–myosin was also found to be adsorbed, myosin in 0.5 M KCl did not seem to be adsorbed by agarose–ATP.

Both Mg^{2+} and Ca^{2+} appear to activate the splitting of bound ATP by heavy meromyosin to practically the same extent.

We prepared water-soluble derivatives of ATP in which ATP underwent the same chemical modification required for its coupling to agarose but in which the agarose component was absent. Their splitting by heavy meromyosin was also activated by Mg^{2+} though to a lesser extent but actin did not influence this reaction.

Possible relations between our findings and the various stages of the reaction between myosin and ATP, as well as the potential use of columns filled with insolubilized NTPs for the separation and purification of myosin and of its subfragments, are discussed.

INTRODUCTION

The binding to macromolecular matrices of various components of enzymatic complexes, particularly the enzymes themselves, and also of inhibitors and cofactors, has opened new horizons and promising applications in enzymology[1,2]. While many enzymes are immobilized in living cells, most enzymatic studies have limited themselves to water solutions. The newly introduced synthetic "insoluble" matrices, to which biological functions have been bound, bridges the gap between the *in vivo* and soluble *in vitro* systems and their investigation may combine advantages of both approaches: similarly to enzymes in solution, they are relatively simple and controlable and, on the other hand, may, in several respects, better represent immobilized natural systems.

An important enzymatic complex to which the new approach has practically not been applied is that of the mechanochemical transducers responsible for biological

motility. As part of a general study of immobilized components of the actomyosin system[3] we have prepared insolubilized ATP by covalently binding ATP to a cross-linked macromolecular carrier. In addition to serving as an almost universal fuel in practically all biological motile organelles, ATP participates in many other bioenergetic processes and its immobilization might provide us with a new tool for the investigation of these processes, as they take place *in vivo*.

In the following work we had in mind two purposes: to try and learn more about the various stages of the mechanism of interaction between heavy meromyosin and ATP and to explore the potentials of insolubilized ATP in affinity chromatography. For these purposes we followed the binding of heavy meromyosin to agarose–ATP gels prepared by us, investigated the enzymatic cleavage of the bound ATP by heavy meromyosin and examined the possibility of eluting bound heavy meromyosin by soluble ATP. We have paid special attention to the effect of calcium and magnesium ions on these three processes which might correspond to some of the stages of the raction of binding and splitting of ATP by myosin.

EXPERIMENTAL

Materials

Sepharose 2B was purchased from Pharmacia Co., Sweden; ATP was a Sigma product.

Preparation of adipic acid dihydrazide

100 ml diethyl adipate, 200 ml hydrazine hydrate (98%) and 200 ml ethanol were refluxed for 3 h. The resulting adipic acid dihydrazide was crystallized from an ethanol–water mixture (m.p. 169–171 °C).

Preparation of Sepharose adipic dihydrazide conjugate

Cyanogen bromide activation of Sepharose 2B (hereafter also named agarose) was performed according to Axen *et al.*[4]. The activated Sepharose gel was suspended in 1 volume of a cold saturated solution of adipic acid dihydrazide (approx. 90 g/l) in 0.1 M sodium carbonate buffer, pH 9.5. The reaction was allowed to proceed for 16 h in the cold, with stirring. The gel was washed thoroughly with water and 0.2 M NaCl solution until the washings gave a very slight color reaction with trinitrobenzene sulfonate[5].

Periodate oxidation of ATP

A sodium metaperiodate solution in water was added into a cold neutral solution of ATP to give a final concentration of 0.020 M in ATP and 0.018 M in periodate. The solution was left for 1 h in the dark at 0 °C.

Coupling of periodate oxidized ATP to Sepharose–adipic hydrazide

50 ml Sepharose hydrazide gel were suspended in 50 ml of a solution containing 0.2 M sodium acetate buffer at pH 5 and 150 μmoles of oxidized ATP. The suspension was stirred for 2 h at 4 °C. The gel was washed (with suction) with 1 M NaCl solution, then with 0.001 M EDTA solution and finally with double distilled water. The washed gel was stored at 4 °C or, for longer periods, at −10 °C. In the latter case, vigorous stirring for 2 h after defreezing was needed in order to re-swell the Sepharose.

Characterization of the agarose–ATP gel

(1) Measurement of the decrease in absorbance (at 259 nm) of the coupling reaction supernatant (combined with the washings of the gel) compared to a blank reaction with unoxidized ATP: 1.5 μmoles of oxidized ATP were found to be bound per ml of Sepharose 2B hydrazide gel, while unoxidized ATP did not bind.

(2) Liberation of inorganic phosphate during the oxidation and coupling reactions: this was determined according to Fiske and SubbaRow[6]. No significant amounts of inorganic phosphate were liberated during these stages.

(3) Determination of total bound phosphate content of the agarose–ATP gel was carried out after treatment with $HClO_4$, according to King[7]: 4.5–5 μmoles phosphate were found per ml of the agarose–ATP gel in accordance with the optical absorbance data suggesting the intactness of the triphosphate grouping.

Preparation of soluble derivatives of ATP

(*1*) *Oxidized ATP.* $NaIO_4$ solution was added into a neutral ATP solution to give final concentrations of 0.020 M ATP and 0.022 M $NaIO_4$. The solution was left at 0 °C for 1 h in the dark. 0.05 ml ethylene glycol was then added per ml of oxidized ATP to destroy unreacted periodate.

(*2*) *ATP–adipic acid dihydrazide.* 1.5 g of adipic acid dihydrazide was dissolved in 15 ml 0.25 M sodium acetate buffer at pH 5 and 15 ml of the above solution of oxidized ATP were added. The solution was left for 2 h in the cold and the ATP hydrazide product separated from unreacted adipic acid dihydrazide by precipitation in 5 volumes of cold ethanol. The precipitate was re-dissolved in water and precipitated again in ethanol. The last procedure was repeated. The precipitate was collected by centrifugation and dried over P_2O_5 in vacuum. The resulting rubber-like material was suspended in dry ether and dried again to give a white-yellowish powder.

(*3*) *ATP–hexanoic acid hydrazide.* The procedure was the same as for ATP–adipic dihydrazide. Hexanoic acid hydrazide was prepared similarly to adipic acid dihydrazide.

Characterization of the soluble derivatives of ATP

Thin-layer chromatography was run on Kieselguhr plates containing a fluorescent marker (DC-Karten SI F. Riedee-De Haën Ag Seelze, Hannover) with a solvent system of *n*-propanol–water (2:1, v/v). Detection of the spots was done (a) by a an ultraviolet lamp; (b) by spraying trinitrobenzene sulfonate test reagents in order to detect hydrazide-containing spots.

The oxidized ATP preparation appeared to be composed of one compound only. In the ATP–adipic acid dihydrazide product a major and a minor spot were detected, both moving slower than oxidized ATP and containing hydrazide groups. Some unseparated adipic acid dehydrazide could also be detected. The ATP–hexanoic hydrazide product moved faster than oxidized ATP and appreared to be quite homogeneous yet contained traces of unseparated hexanoic acid hydrazide.

Preparation of myosin

This was performed according to Azuma and Watanabe[8], using the back muscles of New Zealand white rabbits.

Preparation of heavy meromyosin

The first steps of the procedure published by Tokuyama *et al.*[9] were followed. The crude heavy meromyosin obtained after separation of light meromyosin by centrifugation was used in all our experiments without further purification. Phosphate buffers were replaced by imidazole buffers at pH 7.0.

Adsorption and elution of heavy meromyosin from the agarose–ATP resin

In these experiments batchwise adsorption and elution of heavy meromyosin from the ATP resin were conducted.

(*1*) *Adsorption.* The reaction suspension (3 ml) contained 1 ml Sepharose–ATP gel, 1.6 mg heavy meromyosin and was 0.01 M in imidazole buffer, pH 7. The experiments were carried out in the presence of varying concentrations of Ca^{2+}, Mg^{2+} or EDTA.

15 min after the addition of the last component (heavy meromyosin), during which time adsorption was allowed to proceed at 0 °C with stirring, a 1-ml aliquot was withdrawn and after centrifugation, the ATPase activity and the protein concentration[10] were determined in the supernatant. As blanks, mixtures containing water instead of Sepharose–ATP were checked and in some cases, suspensions of Sepharose–adipic acid hydrazide instead of Sepharose–ATP served as additional blanks.

(*2*) *Elution.* To the suspension left after removal of the 1-ml aliquot, concentrated ATP (or ADP) solution was added to give a final concentration of 5 mM nucleotide. After 15 min stirring at 0 °C, the ATPase activity and the protein concentration of the supernatant were measured.

ATPase and NTPase assays of soluble nucleotides

(a) The reaction mixture (3 ml) was 0.05 M in KCl, 1–4 mM in $CaCl_2$ or $MgCl_2$, 2.5 mM in nucleotide and contained 0.03–2.5 mg myosin or heavy meromyosin. The concentration of the ATP analogues was estimated to be 2.5 mM assuming they were pure. The reaction was followed pH-statically, at pH 7.4, with 0.02 M NaOH as titrant. Routine ATPase assays were performed as above with 4 mM $CaCl_2$ as activator.

(b) The assay solution of 3 ml was 0.50 M in KCl, 4 (or 2) mM in EDTA and 2.5 mM in ATP and contained 0.03–0.5 mg of heavy meromyosin or myosin. The pH of reaction was kept at 7.4 in a pH-stat and the titrant was 0.02 M NaOH. This assay enabled the determination of heavy meromyosin or myosin ATPase in the presence of low concentrations of Mg^{2+} (up to about 0.3 mM Mg^{2+}) in the adsorption and elution experiments.

Determination of ATPase activity of heavy meromyosin with Sepharose–ATP as substrate

The reaction suspension of 10 ml volume was 15 mM in imidazole buffer, pH 7, and contained 4 ml of Sepharose–ATP gel and 8 mg heavy meromyosin. Known amounts of Ca^{2+}, Mg^{2+} or EDTA were added. The suspension was incubated at 25 °C with stirring, aliquots of 1.5 ml withdrawn after predetermined periods of incubation and the Sepharose gel removed immediately by centrifugation. Heavy meromyosin was removed by the addition of 1 vol. of cold 10% trichloroacetic acid and centrifugation. Inorganic phosphate was determined according to Fiske and SubbaRow[6].

RESULTS

Phosphate determination of agarose–ATP (after treatment with $HClO_4$) corresponded to the spectral determination of bound oxidized ATP. This together with the fact that insignificant amounts of inorganic phosphate were found in the supernatant of the agarose–ATP complex reaction mixture as well as in its washings, suggest that the triphosphate portion of ATP remained intact after binding to agarose. We might, therefore, consider agarose–ATP as an insoluble ATP analogue. The results described in the following appear to support this statement.

Adsorption and elution of heavy meromyosin

In Table I we present data on the adsorption of heavy meromyosin by agarosed–ATP and its elution by ATP in solution. The experiments were carried out at 0 °C in order to minimize enzymic activity. As can be seen from Table I, both the binding and the elution by ATP of heavy meromyosin are not much affected by either Ca^{2+} or Mg^{2+} in the range of concentrations utilized nor by the addition of EDTA. Comparison of the percentage decrease in ATPase activity to the corresponding percentage of protein removed from the supernatant due to adsorption by the gel indicates the presence of inactive protein in the heavy meromyosin preparation. It can easily be shown from both the adsorption and the elution data that the weight percent of active heavy meromyosin amounts to an average of 61% in our preparation and that adsorption and desorption do not appear to be accompanied by any inactivation of the heavy meromyosin. The ATPase of the eluted protein was found to be "normal": it was activated by Ca^{2+} but not by Mg^{2+} (ref. 11). Clues for a specific binding of heavy meromyosin to the ATP sites of the gel are the fact that we found no adsorption by the agarose–adipic hydrazide preparation obtained prior

TABLE I

AGAROSE–ATP: ADSORPTION AND DESORPTION OF HEAVY MEROMYOSIN

Cation	*Concn (mM)*	*Adsorption experiments*		*Elution experiments*	
		% decrease in ATPase activity of supernatant due to adsorption on gel	*% decrease in protein content of supernatant due to adsorption on gel*	*% of original activity in the supernatant after elution with ATP*	*% of original protein in the supernatant after elution with ATP*
—	0.00	74	45	67	78
Ca^{2+}	0.25	58	34	71	82
	0.75	69	45	82	85
	1.5	83	51	69	82
	4.0	90	55	71	85
Mg^{2+}	0.33	52	36	67	81
	4.0	—	40	—	75
EDTA	4.0	87	—	68	—

to the binding of the oxidized ATP and the very fact that ATP in solution appears to compete for heavy meromyosin with bound ATP. ADP was also found to be capable of desorbing heavy meromyosin from the gel; thus, the replacement of the 5 mM ATP by a 3 mM solution of ADP, in the presence of 4 mM Ca^{2+}, caused the release of 50% of the adsorbed heavy meromyosin. Finally, as we will show later, agarose–ATP may serve as a substrate to heavy meromyosin, indicating again a specific interaction between them.

Water-soluble polyalanine–myosin, prepared according to Edelman *et al.*[12], was also found to be adsorbed by agarose–ATP. Under the conditions applying in Table I, except that the concentration of ATP used for elution was 4 mM, the ATPase activity of the supernatant was decreased by 82% due to adsorption (in the presence of 4 mM Ca^{2+}). About 60% of the adsorbed activity was released by 4 mM ATP in solution.

On the other hand, intact myosin in 0.5 M KCl did not seem to be adsorbed by agarose–ATP (in the presence of 4 mM Ca^{2+} or 0.3 mM Mg^{2+}) as determined from ATPase measurements.

Cleavage of agarose–ATP by heavy meromyosin

In Fig. 1 some of the kinetic runs in the presence of various concentrations of either Ca^{2+} or Mg^{2+} are presented. The possibility that nucleotide leaked during incubation with heavy meromyosin was checked by measuring the spectral absorption at 259 nm after about 3.5 h. The amount was found to be insignificant. As can be seen from the figure, phosphate liberation in the presence of EDTA was relatively slow and only slightly higher than the spontaneous liberation (in the absence of

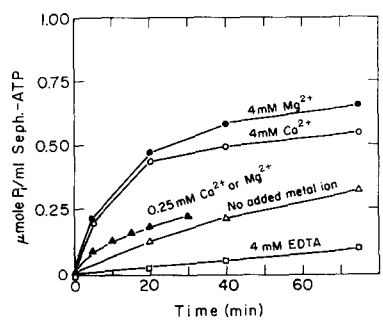

Fig. 1. Phosphate liberation from agarose–ATP by heavy meromyosin *vs* time, in the presence of various concentrations of divalent cations.

heavy meromyosin). Both Mg^{2+} and Ca^{2+} appear to activate the splitting of bound ATP by heavy meromyosin, contrary to soluble ATP which is known to be activated by Ca^{2+} and inhibited by Mg^{2+}. Actually Mg^{2+} sometimes appear to be even better activators than Ca^{2+} at the same concentration. At 1.5 mM and 10 mM, activation by both ions was about the same as in 4 mM and after 3.5 h, some 40–50% of the total bound ATP was cleaved. At a concentration of 20 mM, the activation of both ions was slightly lower than at 4 mM. The initial rate of bound ATP splitting at 4 mM divalent ion concentration was about 0.05 μmole P_i per ml Sepharose–ATP per min.

Our heavy meromyosin preparations were checked and found to behave "normally", *i.e.* they exhibited Ca^{2+} activation and their ATPase activity was inhibited by Mg^{2+}.

NTPase activity of soluble derivatives of ATP

At this point the question has been raised whether this sharp difference between the behavior of bound and free ATP with respect to metal ion activation was associated with micro-environmental effects of the macromolecular matrix or with a chemical modification of ATP as a result of periodate oxidation and/or binding to an adipic dihydrazide residue (or both). In order to clarify this point we prepared soluble derivatives of ATP in which the agarose macromolecular component was absent and in which ATP has been oxidized by periodate and either bound or not bound to a di- or monofunctional C_6 hydrazide. These products have been examined with respect to their capability to be split by heavy meromysin (and myosin) in the presence of Ca^{2+} or Mg^{2+} (as well as EDTA and EDTA +KCl).

In Figs. 2B–2D we have plotted the initial rates of NTP splitting as functions

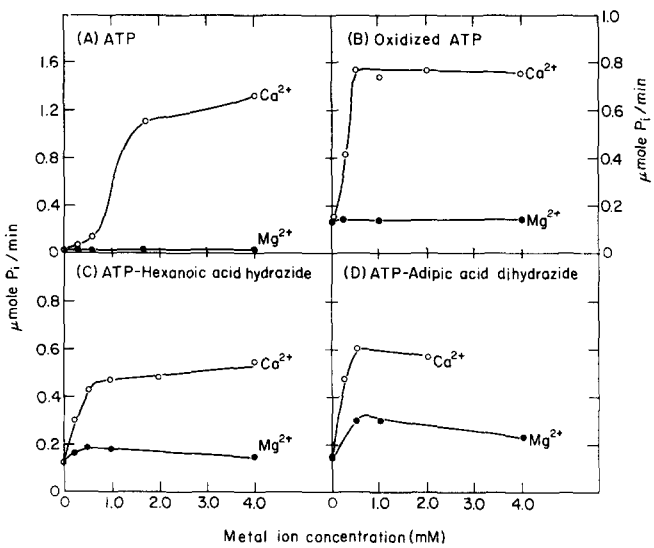

Fig. 2. Initial rates of hydrolysis by heavy meromyosin of ATP and of its water-soluble derivatives as functions of Ca^{2+} or Mg^{2+} concentration. Rates calculated per 1.5 mg heavy meromyosin.

of metal ion concentration for the various ATP derivatives. For comparison we have also plotted in Fig. 2A our determination of ATP splitting by heavy meromyosin as function of Ca^{2+} and Mg^{2+} concentration. It appears that the difference between Mg^{2+} and Ca^{2+} becomes less and less pronounced as we proceed from ATP to oxidized ATP, then to the hydrazide derivatives of oxidized ATP and it finally practically disappears in the oxidized ATP bound by hydrazone formation to agarose (see Fig. 1). Myosin under identical conditions behaved similarly to heavy meromyosin with the soluble analogues as substrates. The Ca^{2+} activation profile of heavy meromyosin by the three ATP derivatives looks quite different from that of ATP

and maximal activation for similar concentrations of the NTPs occurs at significantly lower Ca^{2+} concentrations. In order to check whether the heavy meromyosin has not been irreversibly affected by the soluble ATP derivatives and/or impurities originating from their preparation reaction we added equivalent amounts of ATP to the assay mixtures. Activation by Mg^{2+} disappeared and the system behaved as if the ATP derivative was absent. Myosin ATPase which is known to be activated by EDTA (2 mM) at high KCl concentration (0.5 M) was found to be negligible under these conditions when ATP was replaced by any of our soluble ATP derivatives. When ATP was added to an assay mixture containing myosin, EDTA, KCl and a soluble ATP derivative, phosphate liberation took place at a rate which roughly corresponded to that observed in the usual EDTA–KCl activation of myosin ATPase.

DISCUSSION

After some unsuccessful attempts to demonstrate specific interactions between heavy meromyosin and various other preparations of immobilized ATP, the agarose–ATP described above proved to be capable of binding heavy meromyosin and to be enzymatically split by it, releasing inorganic phosphate. As our experiments (Table I) implicate, active heavy meromyosin appears to be specifically bound from a mixture containing other protein species. This suggests the possibility of utilizing immobilized ATP for the purification of heavy meromyosin and possibly also other active myosin derivatives such as myosin subfragment-1 and polyalanine–myosin. Our attempts to batchwise adsorb myosin at a high ionic strength to agarose–ATP failed, yet we hope to separate myosin from other protein components during its preparation from motile systems by using agarose–ATP filled columns. In order to be able to use such columns economically it is clear that adsorption should be carried out, as we did, under conditions at which the NTPase activity is minimal *i.e.* at low temperature and in the presence of EDTA to minimize splitting of bound ATP or utilizing another immobilized nucleotide which binds heavy meromyosin without being split by it. Pyrophosphate might prove to be the most practical eluent. It would be interesting to find out whether immobilized ATP can distinguish between enzymatically active and inactive heavy meromyosin or myosin subfragment-1 with respect to adsorption and elution, thus enabling further purification and attainment of maximal specific activity.

The chemical modification of ATP, required for its coupling to agarose probably plays a major role in diminishing the gap between the extent of Mg^{2+} and Ca^{2+} as is evident from the behavior of the soluble ATP derivatives we have prepared. However one cannot exclude the possibility that micro-environmental effects of the macromolecular matrix help further in the same direction, thus making Mg^{2+} activation as strong as Ca^{2+} activation. Our findings are reminiscent of a recent report by Liu-Osheroff and Guillory[13], who attached heavy meromyosin subfragment-1 to a cellulose ion-exchange matrix and found it to be activated by both Ca^{2+} and Mg^{2+}. The Mg^{2+}-activated ATPase was inhibited by Ca^{2+} or by higher concentrations of Mg^{2+}. The authors proposed that the covalent binding of heavy meromyosin subfragment-1 either provides the enzyme with a new active-site structure or with a new environment resembling in some way that existing on complexing with actin. It is interesting that Mg^{2+} becomes an activator when either heavy meromyosin sub-

fragment-1 or ATP are bound. According to Lymn and Taylor[14] the desorption of the hydrolysis products of ATP from the active site(s) of myosin is the rate determining step of the ATPase reaction. This desorption is believed to be slowed down by Mg^{2+} and accelerated by Ca^{2+}. It thus appears that the modification of either the active site or the substrate, as was carried up by Liu-Osherof and Guillory[13] and by ourselves, creates a new environment for the decomposition products of ATP which facilitates their desorption in the presence of Mg^{2+}.

The binding of ATP, at least in its soluble derivatives, caused another major change in its behavior: even though it could still undergo splitting by heavy meromyosin it seems to have lost its capability of serving as the "fuel" for the mechanochemical transformation characteristic of the actomyosin complex: unpublished experiments indicate that actin did not influence the Mg^{2+}-NTPase of myosin in the presence of the soluble derivatives; moreover, we failed in our attempts to bring about either superprecipitation of actomyosin or shortening of myofibrils (under the microscope) by adding any of our soluble ATP derivatives under conditions at which ATP itself was effective in both cases. The chemical modification of ATP, which still enabled it to be split by heavy meromyosin, seems to have caused the uncoupling of mechanochemical transduction so that the chemical process could not lead any more to a mechanical change. Tonomura *et al.*[15] have prepared various analogues of ATP, which were found to be incapable of causing the superprecipitation of actomyosin or the contraction of myofibrils in spite of the fact that they exhibited NTPase activity.

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