

ATPase Activity and Light Scattering of Acto-Heavy Meromyosin: Dependence on ATP Concentration and on Ionic Strength

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Actomyosin ATPase, Heavy Meromyosin, Rigor Links

1. The dependence on ATP concentration of ATPase activity and light scattering decrease of acto-HMM could be described at very low ionic strength by one hyperbolic adsorption isotherm with a dissociation constant of 3×10^{-6} M. Hence the increase of ATPase activity was paralleled by a decrease in light scattering. At higher values of ionic strength ATPase activity stopped rising before HMM was completely saturated with ATP. Higher ionic strength prevented ATPase activity from further increasing when the rigor links (links between actin and nucleotide-free myosin), which have formerly protected the ATPase against the suppressing action of higher ionic strength, have fallen below a certain amount. This protecting influence of rigor links did not require tropomyosin-troponin.

2. For complete activation of ATPase activity by actin less actin was needed when HMM was incompletely saturated with ATP than when it was completely saturated with ATP.

3. The apparent affinity of ATP to regulated acto-HMM (which contained tropomyosin-troponin) was lower than to unregulated acto-HMM (which was devoid of tropomyosin-troponin). In the presence of rigor complexes (indicated by an incomplete decrease of light scattering) the ATPase activity of regulated acto-HMM was higher than that of unregulated acto-HMM. At increasing ATP concentrations the ATPase activity of regulated acto-HMM stopped rising at a similar degree of saturation with ATP as the ATPase activity of unregulated acto-HMM at the same ionic strength.

Introduction

A number of substances are known as "interaction inhibitors"^{1,2} which prevent the combination of actin and myosin in the presence of ATP (or other nucleotides) and therefore inhibit actomyosin ATPase. These factors include polyanions, urea² or high ionic strength^{3,4} and the physiological interaction inhibitor tropomyosin-troponin. They all have in common that their degree of inhibition depends on the concentration of ATP^{1,4-7}. Their inhibiting capacity is highest at high concentrations of ATP and decreases with decreasing ATP concentrations. In the particular case of tropomyosin-troponin, Weber and coworkers assumed (based on studies with HMM) that tropomyosin-troponin (in the absence of Ca^{2+}) inhibits only as long as myosin is highly saturated with ATP⁸⁻¹⁰. At lower ATP concentrations "rigor links" (links between nucleotide-free myosin and actin) should exist which were believed to counteract dissociation and hence

inhibition by tropomyosin-troponin. A similar way of arguing was used in a study of the interaction of myosin and actin at different concentrations of ATP and different values of ionic strength⁴, but as straightforward as this interpretation is, it suffers from the fact that the "rigor complexes" were only deduced from the low prevailing concentrations of ATP without being demonstrated by direct means. This paper tries to correlate measurements of light scattering of acto-HMM (which should reveal the formation of acto-HMM complexes with a high light scattering intensity) with measurements of ATPase activity at different concentrations of ATP and different values of ionic strength. Measurements of light scattering and of ATPase activity of acto-HMM at the low range of ATP concentrations used in this paper, have formerly been done by Tonomura and coworkers¹¹⁻¹³ but these workers did not analyze the influence of ionic strength on the relation between light scattering and ATPase activity.

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Abbreviations: HMM, heavy meromyosin; acto-HMM, acto-heavy meromyosin; EGTA, ethyleneglycol-bis(2-aminoethyl)-N,N'-tetraacetic acid; PEP, phosphoenol pyruvate; PK, pyruvate kinase.

Enzymes: ATPase:ATP:phosphohydrolase, EC 3.6.1.3; pyruvate kinase:ATP:pyruvate phosphotransferase, EC 2.7.1.40.



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Experimental Procedures

The preparation of proteins and the measurement of ATPase activity have been described elsewhere^{4,14}. In unregulated acto-HMM (this term was introduced by Weber and coworkers⁸⁻¹⁰) the actin part of acto-HMM is devoid of the regulatory proteins tropomyosin-troponin whereas in regulated acto-HMM these proteins are present. When no special remark was made acto-HMM was always unregulated.

Measurements of light scattering and ATPase activity have been done with aliquots of the same assay. These assays contained besides the components indicated in the figures 1.5 mM PEP and 0.04 mg/ml pyruvate kinase. Pyruvate kinase was suspended in 3.2 M ammonium sulfate, therefore all assays contained in addition to the ionic conditions indicated in the figures 0.012 M $(\text{NH}_4)_2\text{SO}_4$. This relatively high "background ionic strength" was the reason for the relatively low ATPase activity (100%

was in the range of $0.1 \mu\text{mol Pi} \times \text{mg HMM}^{-1} \times \text{min}^{-1}$). The concentration of MgCl_2 was always 1 mM, the pH (Tris-maleate buffer 20 mM) was 7.2; all measurements have been done at room temperature.

Light scattering (at 90° to the incident light) has been measured with a Hitachi-Perkin-Elmer Fluorescence Spectrophotometer MPF-2A at 400 nm. The decrease of light scattering which was observed after the addition of ATP to the cuvette is expressed in %. The decrease which was given by 1 mM ATP and MgCl_2 (this decrease was equal at 5 mM and 600 mM KCl) has been taken as 100% and was determined in every experiment. The adsorption isotherms which were fitted (by taking the respective "dissociation constants" and maximal values obtained from reciprocal plots of the experimental values) to the measured values had sometimes maximal values which were higher than the 100% value measured in the way indicated above.

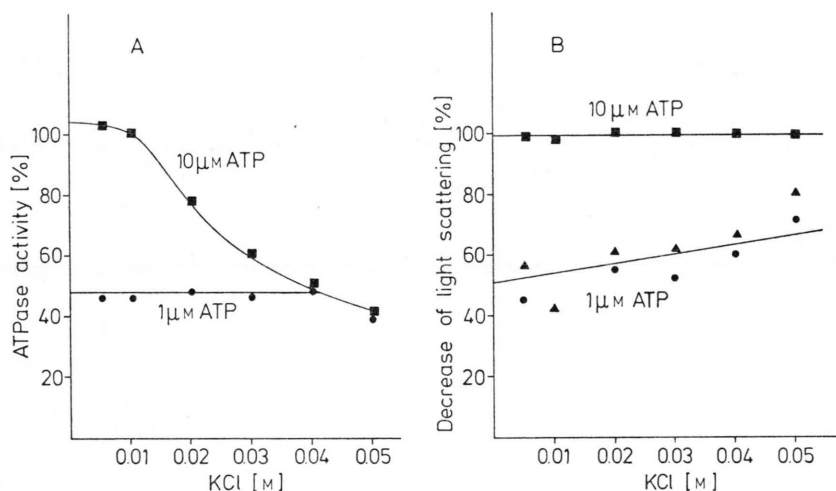
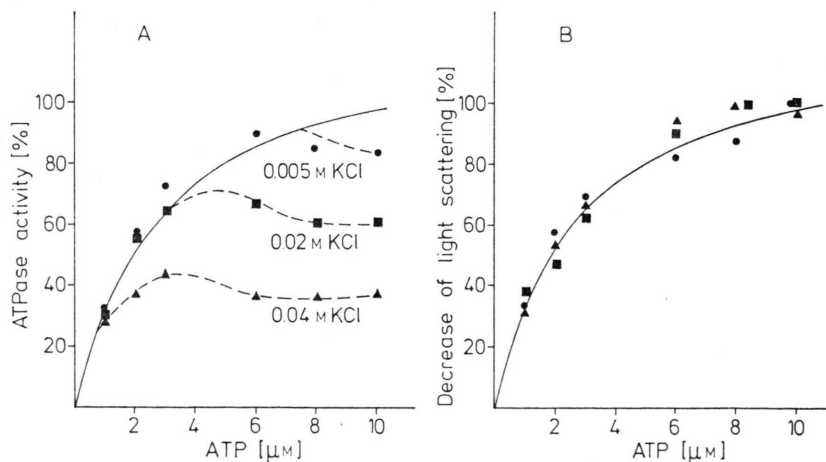


Fig. 1. ATPase activity and light scattering of acto-HMM at different KCl concentrations and at two concentrations of ATP. ■, 10 μM ATP; ●, ▲, two different measurements at 1 μM ATP. 0.22 mg/ml HMM; 0.32 mg/ml actin.

Fig. 2. Dependence of ATPase activity and light scattering of acto-HMM on the concentration of ATP at three different concentrations of KCl. The full line in A and B is a rectangular hyperbola (adsorption isotherm) with a K_D of 2.8×10^{-6} M and a maximal value of 125%. ●, 0.005 M KCl; ■, 0.02 M KCl; ▲, 0.04 M KCl. — 0.26 mg/ml HMM, 0.35 mg/ml actin.



Results

ATPase activity and light scattering of unregulated acto-HMM at different values of ionic strength

In Fig. 1 it is shown that the relation between ionic strength and acto-HMM ATPase activity depended on the concentrations of ATP. At a concentration of $1\ \mu\text{M}$ ATP the ATPase activity did not change in the ionic strength range investigated, whereas at an ATP concentration of $10\ \mu\text{M}$ the ATPase activity was considerably higher at lower values of ionic strength than at higher values. From Fig. 1 B it can be seen that in $10\ \mu\text{M}$ ATP the light scattering decrease after the addition of ATP was already maximal, whereas at an ATP concentration of $1\ \mu\text{M}$ the decrease was incomplete.

The influence of ATP concentration and ionic strength on ATPase activity and light scattering is more explicitly shown in Fig. 2. The decrease of light scattering (Fig. 2B) and the degree of ATPase activity (at the lowest value of ionic strength) can be described by virtually identical curves. Obviously the decrease of light scattering as well as the ATPase activity are both means of measuring the saturation of acto-HMM with ATP. At higher values of ionic strength one should expect that the values of ATPase activity should be reduced at all concentrations of ATP since increasing ionic strength decreases the apparent affinity of actin to myosin in the presence of ATP¹⁵. The picture actually seen in Fig. 2, however, reveals that the values of light scattering decrease were in this experiment independent of ionic strength and that the corresponding values of ATPase activity lie initially at the same curve irrespective of ionic strength. The ATPase activity, however, did not necessarily increase to its (extrapolated) 100% value but stopped rising when a particular degree of saturation of acto-HMM with ATP has been reached. The higher the ionic strength the lower was the ATP concentration at which the ATPase activity stopped rising.

At low concentrations of ATP (when light scattering has only partially decreased indicating a low degree of saturation of acto-HMM with ATP) the suppression of ATPase activity of acto-HMM by higher ionic strength was delayed until the degree of saturation of acto-HMM with ATP exceeded a specific ionic strength dependent value. This could at least qualitatively be accounted for if increasing ionic strength would increase the apparent affinity

of ATP to acto-HMM thus leading to a compensation of the reduced actin affinity. That increasing ionic strength might increase the apparent affinity of ATP to acto-HMM in at least some cases can be supposed from Fig. 1B which indeed shows a small increase of acto-HMM dissociation with increasing ionic strength. However, it seems rather unlikely that such a compensation would give exactly the curves depicted in Fig. 2A and that it would quantitatively explain the independence of ionic strength in $1\ \mu\text{M}$ ATP shown in Fig. 1A. It therefore seems more reasonable to assume that when ATP concentration is low and when rigor links prevail the apparent affinity of actin to HMM is higher (thus overriding the suppressing action of increasing ionic strength) than when ATP concentration is high (cf. also¹⁵). That this is a reasonable assumption is shown in the next section.

The influence of ATP concentration on the saturation of HMM with actin

Fig. 3 shows the activation of HMM ATPase by increasing amounts of actin. The maximal ATPase activity that was possible at the respective concen-

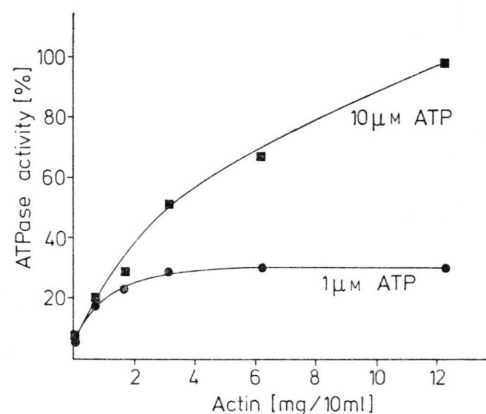


Fig. 3. Activation of HMM ATPase by increasing concentrations of added actin at two different concentrations of ATP. 0.005 M KCl, 0.21 mg/ml HMM.

tration of ATP was attained at a lower actin concentration when ATP was present at a concentration of $1\ \mu\text{M}$ than when it was present at a concentration of $10\ \mu\text{M}$. Hence the apparent affinity of actin to HMM during ATP splitting is higher at an ATP concentration at which a high proportion of rigor complexes can be expected. This suggests that there is some sort of functional connection (of "cooperativity") between rigor complexes and the rest of the

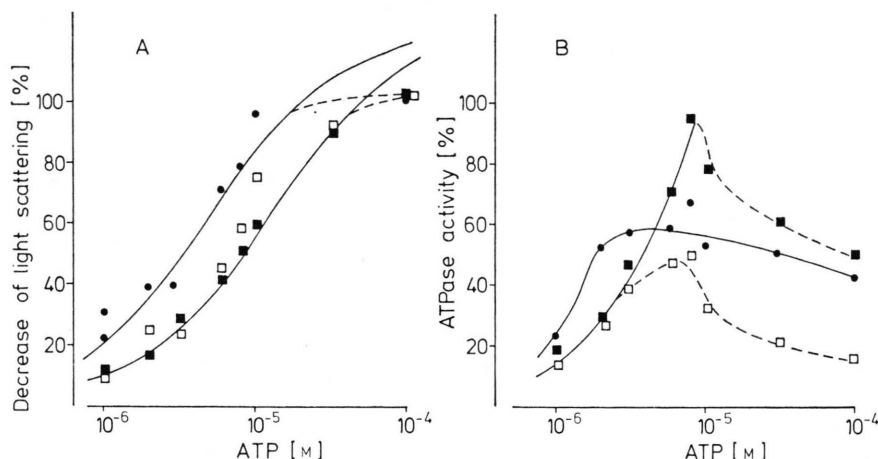


Fig. 4. ATPase activity and light scattering of unregulated and regulated acto-HMM. The full lines in A are adsorption isotherms with a maximal value of 125% and a K_D of 5×10^{-6} M (left) or 12.5×10^{-6} M (right). In B the full line which is fitted to the points of regulated acto-HMM is part of an adsorption isotherm with a maximal value of 310% and a K_D of 2×10^{-5} M. The full line of the ATPase activity of unregulated acto-HMM is drawn by eye. ●, unregulated acto-HMM; ■, regulated acto-HMM, without EGTA; □, regulated acto-HMM with 1 mM EGTA; 0.21 mg/ml HMM; 0.17 mg/ml actin and (in the case of regulated acto-HMM) 0.13 mg/ml tropomyosin-troponin. Tropomyosin-troponin has been prepared as a whole complex [4] and was mixed with actin prior to the addition to HMM. KCl: 0.005 M.

actin filament. This sort of cooperativity did not require the regulatory proteins tropomyosin and troponin since the experiments of Fig. 1 to Fig. 3 have been done with actin which did not contain the regulatory proteins.

The difference which existed between unregulated and regulated acto-HMM will be described in the next section.

ATPase activity and light scattering of regulated acto-HMM

The apparent affinity of ATP to regulated acto-HMM (as revealed by ATPase as well as by light scattering measurements, Fig. 4) was lower than to unregulated acto-HMM. The actual maximal value of ATPase activity of regulated acto-HMM was considerably higher than that of unregulated acto-HMM (Fig. 4B). As can be deduced from the adsorption isotherms of Fig. 4 the actual maximal values of ATPase activity have been reached in unregulated as well as in regulated acto-HMM at about 40% saturation with ATP. EGTA (which did not affect the light scattering of regulated acto-HMM) began to inhibit the ATPase of regulated acto-HMM at about 20% saturation. Whereas the ATPase activity of unregulated acto-HMM decreased only slightly with increasing ATP concentrations (in contrast to observations of Sekiya and Tonomura¹¹, who observed in similar ATPase measurements a consider-

able decrease of ATPase activity of unregulated acto-HMM at higher ATP concentrations), the ATPase activity of regulated acto-HMM became markedly suppressed by increasing saturation with ATP and approached the activity values of unregulated acto-HMM at higher concentrations of ATP.

Discussion

The proportionality between light scattering decrease and ATPase activity

Actin and myosin (or actin and HMM) form in the absence of polyphosphates the actomyosin complex ("rigor complex") which possesses a higher light scattering intensity than the sum of the intensities of actin and myosin alone. Hence the reduction of light scattering intensity after the addition of nucleotides or other polyphosphates indicates the dissociation of actomyosin into actin and myosin or indicates at least a strong reduction of the physical interaction between actin and myosin^{13, 16, 17}.

The decrease of light scattering produced by ATP, which was reported in the present paper could be reasonably well described by an adsorption isotherm (Fig. 2) with a dissociation constant of 3×10^{-6} M. The dependence of ATPase activity of acto-HMM could at lower concentrations of ATP be described by the same adsorption isotherm. This

means that the increase of enzymatically active HMM molecules in increasing concentrations of ATP was proportional to the decrease of acto-HMM complexes with high light scattering intensity. In other words, in the present experiments only "rigor acto-HMM" (and not that part of HMM that actually split ATP) contributed to the high actomyosin light scattering. That acto-HMM, when splitting ATP appears dissociated can be deduced from the viscosity measurements of Szent-Györgyi¹⁸ and has been shown by further viscosity studies by Leadbeater and Perry¹⁹ and Eisenberg and Moos²⁰ and has also been seen in recent spin label experiments^{21, 22} and can further be inferred from²³. Three possibilities may contribute to this phenomenon. 1. The physical interaction between actin and myosin during the course of ATP splitting does not result in a strongly scattering acto-HMM complex. 2. The physical contact between actin and HMM lasts only a short period during the splitting cycle. The rest of the time HMM might be "refractory"^{24, 25} and incapable of binding to actin. 3. The main reason may be that the apparent affinity between actin and myosin is so low that the number of actin and HMM molecules that did interact could not be detected by the method applied. This, however, would imply that in the present experiments the majority of HMM molecules which were combined with ATP and therefore contributed to the decrease of light scattering (since they were dissociated from actin) did not show actin-activated ATPase activity whereas the ATPase activity measurements were based on the actually splitting molecules. Then the identity of the "dissociation constants" inferred from both ATPase and light scattering measurements would mean that the Michaelis constant of actin-activated HMM ATPase was determined by the binding of ATP to HMM and not by the events which follow in the complex reaction chain²⁶⁻²⁸ of acto-HMM ATPase.

The dissociation constant (similar values can be deduced from¹¹) is in reasonable agreement with recent findings concerning the kinetics of myosin and actomyosin ATPase (Bagshaw and Trentham²⁹, Wolcott and Boyer³⁰). The latter authors measured the rate of dissociation of ATP from myosin, that is the reversal of myosin-ATP binding. From their data a binding constant for ATP-myosin binding of about $2 \times 10^9 \text{ M}^{-1}$ (equivalent to a ΔG^0 of -53.1 kJ) can be inferred. In actomyosin this reverse

reaction was estimated to be 2000 times faster than in myosin. This would give (assuming that the forward rate constant remains unchanged) a binding constant for ATP-myosin binding in the presence of actin of about $9 \times 10^5 \text{ M}^{-1}$ (equivalent to a ΔG^0 of -34.7 kJ) which is in the range of the constant that can be deduced from the experiments of the present paper. This means that about 18 kJ of the standard free energy change of the binding of ATP to myosin are used in order to dissociate the rigor links between actin and myosin.

For the dissociation of the rigor links between myosin and regulated (rather than unregulated) actin still more energy seems to be necessary since more ATP was needed to dissociate HMM from regulated than from unregulated actin. This reflects a higher "rigor affinity" between HMM and regulated actin.

The protection by rigor links against the ATPase inhibition of increasing ionic strength

Low ATP concentration counteracted the suppression of ATPase activity in unregulated as well as in regulated acto-HMM. Likewise independent of the presence of the regulatory proteins was the degree of saturation of acto-HMM with ATP at which the ATPase activity stopped rising despite further increase of ATP concentration (Fig. 4). This suggests that the rigor links act directly on the actin filament and not *via* the tropomyosin strand. That binding of HMM to actin is able to alter properties of actin filaments that are devoid of the regulatory proteins has been described with respect to filament flexibility^{31, 32}, behavior of fluorescent^{32, 33} or spin label probes³³. These properties have been altered also in those cases in which less HMM than actin units was present so that HMM must have exerted its influence over more than one actin unit.

Unregulated and regulated acto-HMM differed in two points: a. Equal degrees of saturation of acto-HMM with ATP were attained in regulated acto-HMM at a higher ATP concentration than in unregulated acto-HMM and b. as long as rigor links prevailed (indicated by the incomplete decrease of light scattering, *c.f.* Fig. 4) was the ATPase activity of regulated acto-HMM in the presence of Ca^{2+} higher than that of unregulated acto-HMM at the same degree of saturation with ATP. This may indicate that nucleotide-free myosin ("rigor myosin") has not only a higher affinity to regulated actin

(as has already been discussed) but that rigor links also increase (provided Ca^{2+} has not been removed) the affinity of regulated actin (as compared to unregulated one) to those HMM molecules which contain the nucleotide. Concerning higher ATPase activity of regulated actomyosin as compared to un-

regulated one similar observations^{4, 9, 34-36} and interpretations^{4, 9, 10} can be found in the literature.

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