The Adenosine Triphosphatase Activity of Acto–Heavy Meromyosin. A Kinetic Analysis of Actin Activation*

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ABSTRACT: Using adenosine triphosphatase (ATPase) measurements the interaction of actin with heavy meromyosin was studied in the presence of magnesium at low ionic strength. Linear plots were obtained when, at constant ATP and heavy meromyosin concentrations, the reciprocal of the ATPase activity was plotted against the reciprocal of the added actin concentration. From the intercepts of these plots it was determined first, that the maximum ATPase of the acto–heavy meromyosin complex is about ten times higher than the ATPase of actomyosin, making it similar to the ATPase activity of maximally working muscle; and second, that with magnesium present at low ionic strength the acto–heavy meromyosin is about 100 times more dissociated in the presence of ATP than in its absence. Since an increase in ATP concentration from 0.50 to 2.0 mM did not further decrease the binding between the actin and the heavy meromyosin, it would appear that both the actin and the ATP responsible for dissociating the acto–heavy meromyosin can bind simultaneously to the heavy meromyosin molecule.

This in turn suggests that the binding of ATP to the hydrolytic ATPase site might itself be the cause of the ATP-induced dissociation of the acto–heavy meromyosin.

Despite numerous enzymatic and physical studies on the interaction of heavy meromyosin with actin and ATP[1] at low ionic strength, a number of aspects of this interaction remain quite unclear. Several workers have observed that a maximum ATPase activity of about 0.1–0.5 μmole/(min mg) of heavy meromyosin is reached at roughly stoichiometric ratios of actin to heavy meromyosin (Leadbeater and Perry, 1963; Kominz, 1966); on the other hand, it has been reported that, as the actin concentration is increased to much higher values, the ATPase rate can be as high as 1.5–2 μmoles/(min mg) (Yagi *et al.*, 1965; Sekiya *et al.*, 1967). Another unsolved problem is the occurrence of nearly 20-fold activation of the heavy meromyosin ATPase by actin under conditions where viscosity measurements indicate complete dissociation of the acto–heavy meromyosin complex (Leadbeater and Perry, 1963; Perry *et al.*, 1966; Eisenberg and Moos, 1967).

In the present study we have found that a strictly linear relationship is obtained when, at constant ATP concentration, the reciprocal of the ATPase rate is plotted against the reciprocal of the added actin concentration. From the intercepts of such plots we have determined first, that the maximum ATPase of the acto–heavy meromyosin is approximately ten times higher than the observed ATPase of acto–myosin, making it similar to the ATPase activity in intact muscle under conditions of maximal work, and second, that with magnesium present at low ionic strength, the acto–heavy meromyosin is about 100 times more dissociated in the presence of ATP than in its absence. Furthermore, our data suggest that it might be the binding of ATP to the hydrolytic site itself which causes this marked decrease in the affinity of the heavy meromyosin for actin.

Methods

Actin and heavy meromyosin were prepared by the methods previously used in our laboratory (Eisenberg and Moos, 1967) except that in the preparation of heavy meromyosin, the myosin was digested with trypsin for 5 rather than 4 min. The ATPase was also measured as previously, using an automatic pH-Stat with the sample at 25°. In all cases the reaction was initiated by the addition of heavy meromyosin and the rate was always constant throughout the course of the reaction, as we have previously reported for acto–heavy meromyosin at low ionic strength (Eisenberg and Moos, 1967).

Results and Discussion

We first analyzed the effect of increasing actin concentration on the acto–heavy meromyosin ATPase by directly plotting the ATPase rate against the added actin concentration. As can be seen in Figure 1, there

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[1] Abbreviation used: ATPase, adenosine triphosphatase.

TABLE I: Dependence of Acto-Heavy Meromyosin ATPase on Heavy Meromyosin Concentration.[a]

Concn of KCl (M)	Concn of Heavy Meromyosin (mg/ml)	ATPase Rate (μmoles/(min mg) of heavy meromyosin)
0.08	0.20	0.26
0.08	0.40	0.28
0.04	0.065	0.92
0.04	0.13	0.95

[a] Conditions: 2 mM ATP, 1 mM $MgCl_2$, and 15 mM imidazole buffer (pH 7.0); in 0.08 M KCl samples, actin concentration = 1.1 mg/ml; in 0.04 M KCl samples, actin concentration = 0.97 mg/ml.

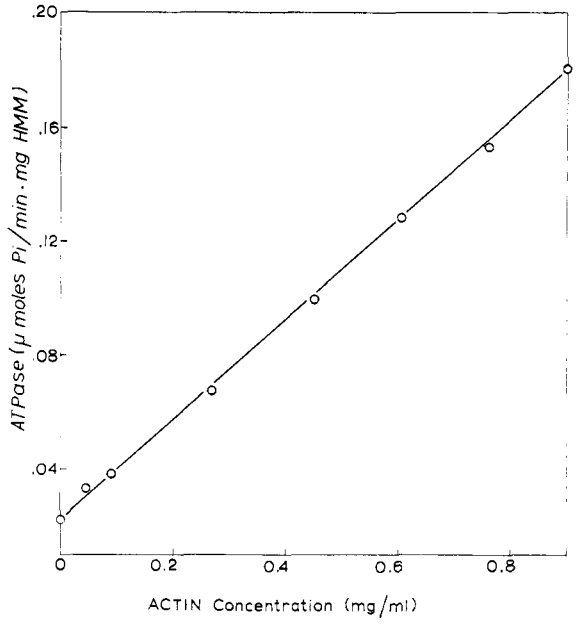


FIGURE 1: Dependence of the heavy meromyosin ATPase on actin concentration. Conditions: 2 mM ATP, 1 mM $MgCl_2$, 11 mM imidazole buffer (pH 7.0), 0.08 M KCl, and 1 mg of heavy meromyosin/ml.

was a steady increase in ATPase activity as the actin concentration was increased so that, even at nearly a 1:1 ratio of heavy meromyosin to actin by weight, the ATPase activity showed no sign of leveling off, a result which is in distinct contrast to observations on actomyosin during superprecipitation where maximum ATPase activity is generally reached at a 4:1 ratio of myosin to actin (Maruyama and Gergely, 1962). Since the rise in ATPase in Figure 1 is essentially linear with increasing actin concentration, much of the heavy meromyosin must remain in the free state even at a 1:1 weight ratio of heavy meromyosin to actin, and therefore the problem remains of determining the true ATPase activity of the acto-heavy meromyosin complex, that is, the ATPase which heavy meromyosin would have if it were completely complexed with actin.

Rather than attempting to determine this ATPase by direct measurement, which, even if it were possible, would require extremely high actin concentrations, it seemed to us more feasible to treat the actin as a simple activator of the heavy meromyosin ATPase, plot the reciprocal of the acto-heavy meromyosin ATPase against the reciprocal of the added actin concentration at constant ATP, and then determine the maximum acto-heavy meromyosin ATPase by extrapolating to infinite actin concentration. This type of plot is only valid, of course, if the heavy meromyosin binding sites on an actin filament are acting independently, and if the free and added actin concentrations can be assumed to be essentially equal. The first of these assumptions is supported by the observation that in the absence of ATP (Young, 1968) or Mg (Rizzino *et al.*, 1968) multiple-equilibrium analysis of actin–heavy meromyosin binding gives a single intrinsic dissociation constant. To verify the second assumption for the conditions used below, we doubled the heavy meromyosin concentration while holding the actin concentration constant at the lowest actin concentrations to be employed. If an appreciable fraction of the added actin were complexed by heavy meromyosin, a change in the heavy meromyosin concentration would alter the free actin concentration and hence would also affect the ATPase per milligram of heavy meromyosin. But Table I shows that neither at 0.04 nor at 0.08 M KCl did doubling the heavy meromyosin concentration change the ATPase per milligram of heavy meromyosin and therefore we are indeed justified in our use of the added actin concentration rather than the free actin concentration in the following reciprocal plots.

To determine the acto-heavy meromyosin ATPase for these plots, the measured ATPase rate was corrected by subtracting the ATPase rate of the total added heavy meromyosin in the absence of actin (0.02 μmole/(min mg) of heavy meromyosin). Subtraction of this ATPase rather than that of the free heavy meromyosin is justified because, for those points where an appreciable fraction of the heavy meromyosin is bound, the velocity is so high that the correction itself is inconsequential.

In Figure 2 we have plotted, for several different conditions, the reciprocal of the acto-heavy meromyosin ATPase activity against the reciprocal of the added actin concentration, and as can be seen this approach is clearly successful, giving linear plots. The intercept on the ordinate gives a measure of the ATPase of the heavy meromyosin at infinite actin concentration, *i.e.*, that of the fully complexed acto-heavy meromyosin, and for all conditions tested this ATPase has a value of about 4 μmoles/(min mg) of heavy meromyosin which is equivalent to 200-fold activation of the heavy meromyosin ATPase by actin. This is much higher than the values of 0.5 μmole/(min mg) or less which are generally observed for the acto-heavy meromyosin ATPase (Leadbeater and Perry, 1963; Kominz, 1966; Perry *et al.*, 1966), and therefore it is likely that under the usual ATPase assay conditions where a 3:1 weight

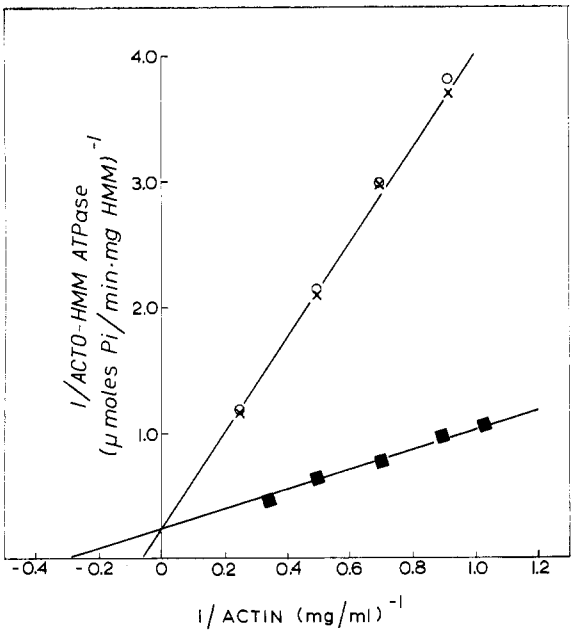


FIGURE 2: Reciprocal plots of acto–heavy meromyosin ATPase *vs.* actin concentration. All samples contained 75 mM imidazole buffer (pH 7.0). (×) 0.08 M KCl, 2 mM ATP, 1 mM $MgCl_2$, and 0.2 mg of heavy meromyosin/ml. (■) 0.04 M KCl, 2 mM ATP, 1 mM $MgCl_2$, 0.065 mg of heavy meromyosin/ml. (○) 0.091 M KCl, 0.5 mM ATP, 0.25 mM $MgCl_2$, and 0.2 mg of heavy meromyosin/ml.

ratio of heavy meromyosin to actin is employed, the acto–heavy meromyosin is, in reality, quite dissociated. This in turn provides at least a partial explanation for the low viscosity of acto–heavy meromyosin which has been observed in the presence of MgATP at low ionic strength (Leadbeater and Perry, 1963; Perry *et al.*, 1966; Eisenberg and Moos, 1967), and it implies that many reported values for the acto–heavy meromyosin ATPase are actually measures of the ATPase of a mixture of both free and actin-bound heavy meromyosin.

Our extrapolated ATPase rate of 4 μmoles/(min mg) for the acto–heavy meromyosin complex is not only higher than the usual rate reported for acto–heavy meromyosin, but it is also about ten times higher than the ATPase rate generally observed for actomyosin during superprecipitation (Maruyama and Gergely, 1962). Interestingly enough, however, it appears to be quite similar to the ATPase rate found in intact working muscle. Kushmerick has found an ATPase rate of roughly 1 μmole/g of muscle per sec for maximally working frog sartorius muscle at 0° (Kushmerick, 1966). Assuming a heavy meromyosin content of 6% in muscle (Hanson and Huxley, 1957) and a sixfold increase in ATPase for the temperature rise from 0 to 25° (Levy *et al.*, 1962), this *in vivo* ATPase is equivalent to an ATPase rate, under our conditions, of 6 μmoles/(min mg) of heavy meromyosin, which compares favorably with our value of 4 μmoles/(min mg) for the acto–heavy meromyosin complex. This agreement between the *in vivo* and *in vitro* rates suggests that the low ATPase rate generally observed in actomyosin might reflect only the relatively disorganized nature of the actomyosin precipitate; with the true ATPase of the actomyosin complex being, in reality, quite similar to the ATPase of the acto–heavy meromyosin complex.

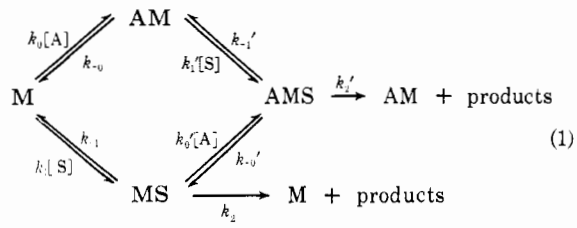
In addition to giving the true ATPase of the acto–heavy meromyosin complex, the double-reciprocal plot technique also yields a separate measure of the binding affinity between the actin and heavy meromyosin under the given conditions of the experiment. It is therefore possible, for the first time, to determine independently how a given agent affects the binding of heavy meromyosin to actin and how it affects the ATPase of the acto–heavy meromyosin complex. This is illustrated for KCl in Figure 2 where the reciprocal plot at 0.08 M KCl (crosses) can be compared with a similar plot at 0.04 M KCl (squares). Although, as we noted above, there is no change in the intercept on the ordinate with a twofold increase in KCl concentration, there is clearly a marked change in the intercept on the abscissa; and therefore we can conclude that ionic strength affects the binding of actin to heavy meromyosin without significantly affecting the ATPase of the acto–heavy meromyosin complex.

From the intercept on the abscissa in Figure 2, we find that at 0.08 M KCl with MgATP present, the apparent dissociation constant for the acto–heavy meromyosin complex is about 2.5×10^{-4} M, assuming a binding ratio of 5×10^4 g of actin/mole of heavy meromyosin (Young, 1968; Rizzino *et al.*, 1968). This is roughly 1/100 as strong a binding of actin to heavy meromyosin as occurs in the absence of ATP (Young, 1968) or Mg (Rizzino *et al.*, 1968) and to investigate this dissociating effect of ATP at low ionic strength, we have applied the double-reciprocal plot analysis. In Figure 2, a reciprocal plot at 0.5 mM ATP–0.25 mM $MgCl_2$ (circles) can be compared with a similar plot at 2 mM ATP–1 mM $MgCl_2$ (crosses), with the ionic strength held constant. As can be seen, in contrast to the effect of KCl, the fourfold change in ATP concentration had no effect whatever on the ATPase activity; that is, neither the extrapolated ATPase of the acto–heavy meromyosin complex nor the binding affinity of the heavy meromyosin to the actin was altered.

This result is consistent with a recent report of Sekiya *et al.* (1967), and also with our previous observation that there is no change in the ATPase rate of acto–heavy meromyosin as the ATP concentration falls during the course of a reaction (Eisenberg and Moos, 1967). It does not imply, of course, that, at a much lower ATP concentration (in the micromolar range), the ATPase would not change as the ATP concentration fell, but it clearly shows that increasing the ATP concentration in the millimolar range has no effect on the binding of actin to heavy meromyosin. We and others have previously suggested that the dissociation of actomyosin by ATP is caused by the binding of ATP to a "dissociating site" on the myosin distinct from the hydrolytic ATPase site (Weber and Portzehl, 1954; Eisenberg and Moos, 1965; Levy and Ryan, 1966). However in the light of our finding that, at high ATP concentration, the addition of further ATP does not affect the actin–heavy meromyosin

binding, we now believe that the dissociating effect of ATP might be explained more easily by simply assuming that the binding of ATP at the hydrolytic ATPase site decreases the affinity of the heavy meromyosin for actin but does not completely prevent the actin–heavy meromyosin binding.

The kinetics of such a model may be considered in terms of the general scheme for the interaction of enzyme, substrate, and modifier described by Botts and Morales (1953), where in our case the enzyme is heavy meromyosin and the modifier is actin (eq 1).



(1)

Here A represents actin, M represents heavy meromyosin, and S represents ATP. It should be noted that, although we have given the substrate as ATP, magnesium is apparently required for both actin activation of the heavy meromyosin ATPase (Maruyama and Watanabe, 1962) and dissociation of the acto–heavy meromyosin by ATP at low ionic strength (Rizzino *et al.*, 1968) so that it may well be that MgATP is the true substrate for heavy meromyosin.

The steady-state rate equation for a general kinetic scheme of this type has been derived by Botts and Morales (1953) and is quite complex. However at high ATP concentration the rate equation reduces to the following limiting form (Botts, 1958)

$$\frac{1}{(v - k_2)} = \left(\frac{1}{(k_2' - k_2)K_0'}\right)\left(\frac{1}{[A]}\right) + \frac{1}{(k_2' - k_2)}$$

where $(v - k_2)$ equals the rate of ATP hydrolysis/mg of heavy meromyosin minus the ATPase rate of heavy meromyosin in the absence of actin, and K_0' equals k_0'/k_{-0}'. This equation is clearly consistent with our observation that, at high ATP concentration, plots of $1/(v - k_2)$ *vs.* 1/[actin] are linear and are unaffected by a further increase in ATP concentration. Furthermore our model can easily account for the actin activation of the heavy meromyosin ATPase if we assume that $k_2' \gg k_2$, and for the dissociating effect of ATP if we assume that $K_0' \ll K_0$, where $K_0 = k_0/k_{-0}$, *i.e.*, that the binding affinity of actin to the heavy meromyosin–ATP complex is much less than the binding affinity of the actin to the heavy meromyosin alone.

Therefore, although we cannot rule out the possibility that there is a separate dissociating site for ATP on the heavy meromyosin, all of our kinetic data at this point are consistent with the simple postulate that dissociation of the acto–heavy meromyosin is caused by the binding of ATP at the hydrolytic site. This view is further supported by our recent finding that subfragment 1, a further tryptic digestion product of heavy meromyosin, which has been reported to have only one binding site for nucleotide (Young, 1967), acts in a similar manner to heavy meromyosin, that is, in the presence of magnesium the actin activates the ATPase of the subfragment 1 (Eisenberg *et al.*, 1966) while, at the same time, the ATP markedly dissociates the acto–subfragment 1 complex at low ionic strength (Eisenberg and Moos, 1968).

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Added in Proof

Since this paper was submitted Szentkiralyi (1967) has also reported linear double-reciprocal plots for the actin activation of the heavy meromyosin ATPase.

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