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# Binding of Adenosine Triphosphate to Myofibrils during Contraction and Relaxation†

Koscak Maruyama‡ and Annemarie Weber*

ABSTRACT: It is possible to measure ATP binding to myofibrils under conditions of rapid hydrolysis, because free ATP can be maintained phosphorylated with the creatine phosphate–kinase system. Nevertheless, it is very likely that not all of the nucleotide is ATP but that some or most of it is ADP bound to the three or possibly four nucleotide-containing enzyme–substrate and enzyme–substrate–cofactor intermediates that must exist. Nucleotide binding to myofibrils can be described fairly accurately by two reciprocal $K_M$ values, one for 25% and the second for the remainder of the sites. Removal of calcium, while apparently not affecting the first, increased the second $1/K_M$ about fivefold. The total number of binding sites was equal to the total number of myosin "heads" and was the same in the presence and absence of calcium. This latter observation is not compatible with the concept that during relaxation ATP is bound to a special "relaxing" site from which it is displaced in the presence of calcium. Arguments are presented in favor of explaining the change of $K_M$ of ATP binding on calcium removal by the reduction of the rate of ATP hydrolysis through inhibition of the reaction actin + myosin ~ → A~M (force-generating complexes). The difference in the $K_M$ of 25% of the total sites may be explained in a number of ways without it being possible at this time to decide which one is correct. Among the possibilities is a form of negative cooperativity that differs from the usual mechanism and was therefore modeled.

Muscle requires ATP not only for contraction but also for relaxation and the maintenance of the resting state. After removal of ATP, most of the actin and myosin molecules form bonds with each other, causing the muscle to go into rigor. By contrast, during rest, muscle is very extensible because actin and myosin filaments are completely dissociated from each other. ATP is needed to break the complex between actin and myosin—an action for which polyphosphates, such as nucleoside di- and triphosphates and inorganic pyrophosphate seem to be specific. However, ATP causes dissociation of the actomyosin complex not only during relaxation but also during shortening. The data by Lymn and Taylor (1971) indicate that dissociation follows immediately the binding of ATP to actomyosin. The dissociated state is transitory during contraction: it is terminated by the formation of force-generating links between activated myosin and actin. During relaxation dissociation is permanent, because this recombination of actin and myosin is prevented by calcium-free troponin (Ebashi and Ebashi, 1964; Ebashi *et al.*, 1968). However, troponin can prevent the formation of active actin–myosin links only if sufficient ATP is present. At low ATP, in spite of the removal of calcium, tension is developed (Reuben *et al.*, 1971; White, 1970), shortening and syneresis take place (Weber and Herz, 1963; Levy and Ryan, 1965; Endo, 1964), and ATP hydrolysis is activated by actin (Weber and Herz, 1963; Weber, 1969). It appears then, that ATP is not only responsible for the breaking of the complex between the two proteins, but, in addition, in some way is involved in the reaction by which calcium-free troponin prevents the formation of an active actin–myosin complex. We wondered (Weber *et al.*, 1964) as did others (Levy and Ryan, 1965) whether ATP may bind to a relaxing site, in addition to the hydrolytic site. Therefore, we wanted to know the total number of binding sites for ATP in the myofibril, and find out whether under relaxing conditions (in the absence of calcium and at high ATP) more ATP is bound than in the presence of calcium.

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Such information on the role of ATP in the functioning myofibril is not obtained from studies of ATP binding sites of isolated myosin (Schliselfeld and Barany, 1968; Lowey and Luck, 1969; Kiely and Martonosi, 1969; Nauss *et al.*, 1969; Murphy and Morales, 1970) nor can it be acquired with the methods used for isolated myosin, such as analogs (ADP, inorganic pyrophosphate) or conditions of greatly reduced ATPase activity. Instead we measured the binding of $^{14}C$-labeled ATP under optimal conditions for contraction and relaxation, maintaining the level of free ATP constant by rapidly rephosphorylating the hydrolyzed ATP with creatine phosphate and kinase.

We found that the total number of binding sites in the myofibril was the same during relaxation and contraction and that it was equal to the number of myosin heads in the myofibril. Nevertheless, at low concentrations of ATP, removal of calcium caused the nucleotide binding to increase in a manner which could be formally described (according to the Scatchard plots) as an increase in the ATP affinity. However, if one takes into account all the recent kinetic data (Lymn and Taylor, 1970, 1971), it becomes obvious that a significant fraction of the bound isotope must be present as ADP bound to high energy intermediates of the enzyme–substrate and enzyme–substrate–cofactor complexes. A sufficient number of kinetic constants have been described recently by Lymn and Taylor (1970, 1971) as well as Eisenberg and Moos (1968, 1970) and Szentkiralyi and Oplatka (1969) to set up a likely reaction scheme and to estimate changes in the steady-state distribution of the various intermediates under different conditions.

With such calculations we were able to show that the cause for the increased ATP binding after calcium removal need not be an increase of the affinity of the binding site for ATP. Instead, it may have resulted from changes in the steady-state distribution of intermediates and a reduction in the flux rate subsequent to inactivation of actin by calcium-free troponin.

Some of the results described here have been summarized previously (Weber, 1970).

## Experimental Section

*Methods.* Protein preparations. Myofibrils were prepared as previously described (Weber *et al.*, 1963). Trypsin treatment of myofibrils was performed according to Ebashi and Ebashi (1964).

ATP binding was determined at 25°, in a medium of the following composition: 4 mg/ml of myofibrillar protein–10 mM imidazole (pH 7.0)–1.0 mM $MgCl_2$–creatine phosphate in concentrations varying between 5 and 10 mM–0.6 mg/ml of kinase–KCl to adjust the ionic strength to 0.1–either 2.0 mM EGTA or 40 $\mu$M $CaCl_2$–[$^{14}C$]MgATP. After temperature equilibration, creatine phosphate, kinase, and ATP were added. Following an incubation period of about 20–40 sec, the myofibrils were separated from the medium by 1-min centrifugation in a clinical centrifuge. After complete removal of the supernatant, the pellet volume was determined by weighing. The isotope was extracted from the pellet with 5% trichloroacetic acid and was counted together with a sample of the supernatant at infinite thinness, in a proportional gas flow counter.

The difference between the total isotope in the pellet and the free isotope trapped with the supernatant was taken as the bound isotope. The supernatant trapped in the pellet was estimated from the pellet weight (assuming a specific gravity of 1) minus the dry weight of the protein. The amount of bound ATP was obtained by multiplying the fraction of bound isotope with the quantity of added ATP. The difference between total and bound ATP represents the free ATP.

Protein concentrations were determined by the method of Lowry *et al.* (1951), using a standard curve for myofibrillar protein calibrated by Kjeldahl determination. Control experiments established that not all of the myofibrillar protein was collected in the pellet, but a small fraction, increasing from about 5% at 2–4 $\mu$M ATP to 15% at 60 $\mu$M remained in the supernatant. This was assumed to be mainly actomyosin, because after 1 hr it precipitates and seems to undergo syneresis, and was corrected for in the calculations.

The apparent binding constants for ATP, with the respective number of binding sites, were obtained by a series of approximations using the equations given by Klotz and Hunston (1971).

Syneresis. The extent of syneresis during binding measurements was, before the decantation of the supernatant, estimated from the pellet volume, indicated by the calibrations of the Wassermann tubes used for centrifugation. Its extent was described by the protein concentration in the pellet: it is maximal with 150 mg/ml and zero with 20 mg/ml.

ATPase activity was measured under conditions identical with those for binding, not later than 1 day after each binding experiment. The amount of ATP hydrolyzed was estimated from the amount of creatine liberated during a 30-sec incubation. The reaction was terminated by 2 mM *p*-chloromercuribenzoate, protein was removed according to Somogyi (1945), and creatine was determined by the method of Eggleton *et al.* (1943).

Activity of phosphocreatine kinase by the forward reaction (phosphorylation of creatine by ATP at alkaline pH) was determined according to Bergmeyer (1965).

*Materials.* Creatine phosphate was obtained from CalBiochem, crystalline disodium ATP from Sigma was mixed with [$^{14}C$]ATP from Schwarz, and phosphocreatine kinase was purchased from Boehringer, Mannheim.

## Results

First, we should like to discuss the accuracy of binding data in a system where ATP is hydrolyzed at a rapid rate and the concentration of free ATP is maintained constant with a high concentration of creatine phosphate and kinase. The following problems are considered. (1) What fraction of the total free nucleotide actually consists of ADP, especially in the range of low ATP concentrations? (2) How much free ATP is removed by binding to the kinase? (3) Does kinase bind to myosin so that some of the kinase-bound ADP is counted as myofibril-bound isotope?

The first question may be answered by some calculations valid under conditions where the steady-state concentration of ADP does not exceed 10 $\mu$M, *i.e.*, a value below 0.1 $K_d$ (= 100 $\mu$M, according to Morrison and James, 1965). $K_d$ is defined by eq 1 (where E = kinase), which may be rearranged to

$$K_d = \frac{[E][ADP]}{[EADP]} \quad (1)$$

give $K_d/[ADP] = [E]/[EADP]$. When $K_d/ADP = 10/1$ or more, it follows that $[E] = 0.9\text{–}0.99\,[E_{total}] \cong [E_{total}] \cong$ constant, and consequently $[E]/K_d =$ constant $= K_c$. Rearranging eq 1 and substituting $K_c$ we obtain $[EADP] = K_c[ADP]$. Since the rate of phosphorylation of ADP is given by $-dADP/dt = k_3[EADP]$ we may write $-dADP/dt = k_3K_c[ADP]$, *i.e.*, the rate of ADP removal may be calculated as a pseudo-first-

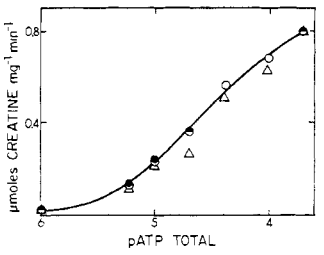



FIGURE 1: The effect of increasing concentrations of kinase on the rate of creatine liberation per milligram of myofibrillar protein with increasing concentrations of MgATP. Myofibrillar protein (4 mg/ml) was incubated under conditions described under Methods with increasing concentrations of MgATP, 10 mM creatine phosphate, and 3 different concentrations of kinase: ●, 2 mg/ml; ○, 1 mg/ml; △, 0.2 mg/ml.

order reaction. With $v_{max}$ (infinite ADP, 10 mM creatine phosphate, 1.0 mM Mg excess over MgADP) of 120–150 μmoles/min per mg of kinase (= 150–200 nmoles/sec per nmole of ADP–kinase complex) the value for $k_3$ is 150–200 $sec^{-1}$; taking 100 μM as the value for $K_d$ (pH 7.0, 4 mM creatine phosphate; Morrison and James, 1965) and using a concentration of kinase of 7.4 μM (= 0.6 mg/ml) the value for $k_3K_c$ is calculated as 11–15 $sec^{-1}$ for all ADP concentrations below 10 μM.

In a system containing ADP-producing myofibrils together with kinase, steady-state conditions are obtained when the ADP concentration has risen to a level where $k_3K_c$[ADP] = μmoles of ATP split $ml^{-1}$ $sec^{-1}$. Adjusting the dimensions of the rates of hydrolysis given by Figure 1, we estimated the following steady-state concentrations of ADP: 3–4 μM for 0.1 mM ATP; 1–1.4 μM for 10 μM ATP; 0.5–0.7 μM for 5 μM ATP; and 0.1–0.18 μM for 1 μM ATP. These estimated levels of ADP may be too high by a factor of 2 because $K_d$ under our conditions (pH 7.0) may have been only 40 μM according to our measurements. These calculations suggest that down to a concentration of 1 μM at least 85% of the ATP is maintained in the phosphorylated state. Furthermore, because ATP concentration and binding are not linearly proportional over most of the concentration range, one would expect the error due to incomplete phosphorylation to stay within 10–12%. Figure 1 illustrates an experiment to test the validity of these considerations. It shows that increasing the concentration of kinase from 2.5 to 25 μM did not increase the rate of creatine liberation by more than 10%.

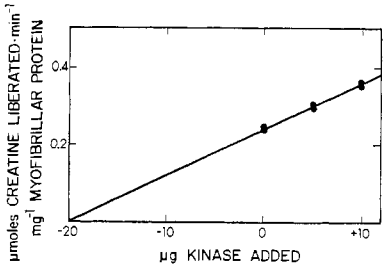



FIGURE 2: Creatine liberation as a function of increasing kinase concentrations in the presence of myofibrils, under conditions where the kinase reaction is rate limiting. Myofibrillar protein (2 mg/ml) was incubated under conditions described under Methods, in the presence of 1.0 mM MgATP and 10.0 mM creatine phosphate, with increasing concentration of kinase. Creatine liberation was measured after 1-min incubation at 25°.

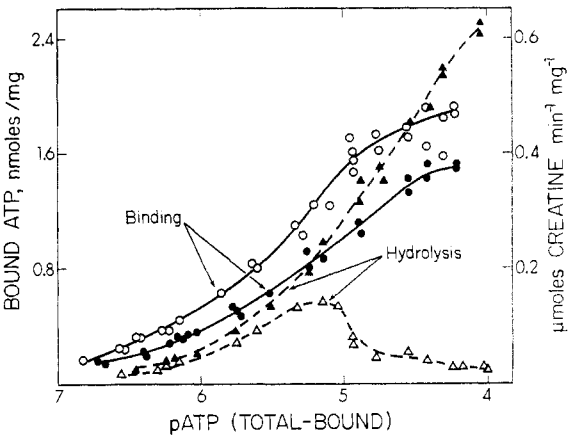



FIGURE 3: Isotope binding and ATP hydrolysis as a function of increasing ATP. The left ordinate indicates bound isotope per milligram of myofibrillar protein. Binding is indicated by circles: open in the absence, closed in the presence of calcium. The right ordinate indicates the rate of creatine liberation per milligram of myofibrillar protein. Corresponding symbols are triangles: open in the absence closed in the presence of calcium.

How much ATP is bound to the ADP site of the kinase? On the basis of our somewhat cursory measurements of ATP binding to kinase we arrived at a binding constant quite similar to the apparent inhibitor constant determined by Morrison and James (1965) which has a value of 870 μM. We found for concentrations of free kinase of 250 and 130 μM a ratio of free to bound ATP of 7.4:2 and 8:1.4, respectively, *i.e.*, according to $K_{D_{ATP}}$ = ([E][ATP])/[EATP], a constant between 740 and 920 μM. Because, during our measurements of ATP binding to myofibrils, the concentration of kinase was only 7.4 μM and 870/7.4 = free ATP/kinase–ATP, the binding of ATP to kinase may be neglected in our experiments.

How much kinase binds to myofibrils? Figure 2 illustrates how we measured the amount of contaminating kinase in our myofibril preparations. It may be seen that myofibrils liberate creatine in the presence of ATP even when no kinase had been added. The rate of creatine liberation increased linearly with the addition of kinase in quantities small enough for phosphorylation to remain the rate-limiting step, rather than ADP production. The amount of contaminating kinase may be read from the point of intersection at the abscissa, assuming that the specific activity of the contaminating and the added kinase are the same. The myofibrils, quite reproducibly, contained about 1–1.5 μg of kinase per mg of protein. We then tested how much additional kinase may be bound to the myofibrils. To that purpose we added 1 mg of kinase to 20 mg of myofibrillar protein, centrifuged, and compared the activity of the kinase in the supernatant with a control not pretreated with myofibrils. The rate of phosphorylation of creatine by ATP was used as an activity test and was found to be 42 μmoles/min after and 38 μmoles/min before pretreatment with myofibrils. Apparently myofibrils do not bind kinase. Even if all of the contaminating kinase, *i.e.*, 0.01–0.02 nmole/mg of myofibrillar protein, had been bound and saturated with ADP the amount would be negligible.

After we had established that it is possible to maintain, with the creatine phosphate–kinase system, a constant level of ATP, we measured the binding of $^{14}C$-labeled ATP to myofibrils. Obviously, such data will not give any information on the state of phosphorylation of the bound nucleotide, *i.e.*, bound ATP is not distinguishable from ADP. During the

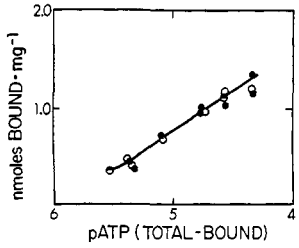



FIGURE 4: Isotope binding by trypsin-treated myofibrils with increasing ATP. Conditions as described under methods except for an ionic strength of 0.05, because of the high solubility of myofibrils after trypsin treatment. Symbols are as in Figure 3.

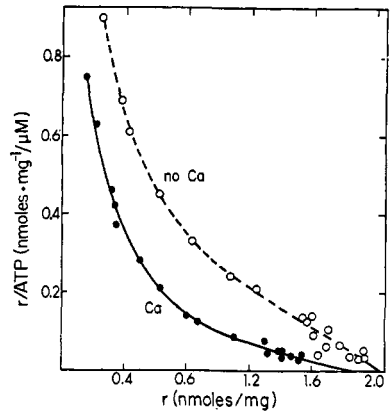



FIGURE 5: Scatchard plot of the binding data of Figure 3 with identical symbols.

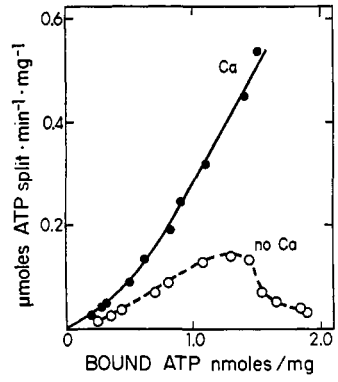



FIGURE 6: Rate of ATP hydrolysis per milligram of myofibrillar protein as a function of bound isotope: ●, in the presence, ○, in the absence of calcium. Data of Figure 3 are replotted.

description of the data we shall, therefore, refer to bound isotope only, without further specification.

Figure 3 shows that, in the range of 0.5–60 $\mu$M free ATP, the removal of calcium from myofibrils increased the binding of isotope. Concentrations of free ATP greater than 60 $\mu$M were not used, because the difference between bound and free isotope in the pellet became too small to obtain significant numbers. Figure 3 also shows the response of myofibrillar ATPase activity to this concentration range of ATP. The extent of syneresis, which under these conditions parallels shortening of the myofibrils (as established by phase-contrast microscopy), changes with increasing ATP in a pattern closely similar to that of the ATPase activity. In the presence of calcium, syneresis and ATPase activity smoothly increase. In the absence of calcium, syneresis and ATPase activity change in a biphasic pattern: activation at low ATP, followed by nearly maximal inhibition at 60 $\mu$M ATP.

Removal of calcium increased ATP binding only when the troponin–tropomyosin system was intact. After trypsin treatment (Ebashi and Ebashi, 1964) caused sufficient proteolysis of troponin to abolish relaxation, this increase in ATP binding was abolished (Figure 4).[1]

Scatchard plots of the data (Figure 5) suggest that removal of calcium does not increase the total number of ATP binding sites. In 3 experiments with a sufficient number of points to permit meaningful extrapolation, the number of sites in the presence and absence of calcium did not differ by more than 0.1–0.2 nmole/mg of protein.

The total number of sites per milligram of protein corresponds to the number of myosin heads (Hanson and Huxley, 1957; Huxley and Hanson, 1957; Lowey *et al.*, 1969).

Figure 5 suggests that the binding of isotope is governed by more than one apparent microscopic binding constant: 25% of the sites seem to bind ATP with a much higher apparent association constant than the remainder. In fact, both binding curves can be fitted very closely with just two binding constants. The value for the first constant and the corresponding number of binding sites are very similar in the presence and absence of calcium with $1.7 \times 10^6$ and $2.1 \times 10^6$ $M^{-1}$, respectively, for 0.5 nmole of binding site per mg of protein (*cf.* Klotz and Hunston (1971) for the meaning of slopes and intercepts). The value for the constant governing the remainder of the sites increased about fivefold on calcium removal, from $5 \times 10^4$ to $2.2 \times 10^5$ $M^{-1}$.

For Figure 6 we plotted the rate of hydrolysis as a function of bound nucleotide. If the curvature of the Scatchard plot (Figure 5) were an indication of two classes of binding sites these classes must also differ in their catalytic constants, because the rate of hydrolysis does not increase in linear proportion with the concentration of bound nucleotide. ATP bound to sites occupied at low concentrations was hydrolyzed more slowly than ATP bound to the remaining 80%. Although this difference is certain, we are not so sure about the significance of some finer detail: it seems that in the presence of calcium the first 0.5 nmole of bound nucleotide was not split at a uniform, but at a steadily increasing, rate, although any contribution by rapidly hydrolyzing sites must have been negligible since the $K_M$ values for both sites differed by 2 orders of magnitude.

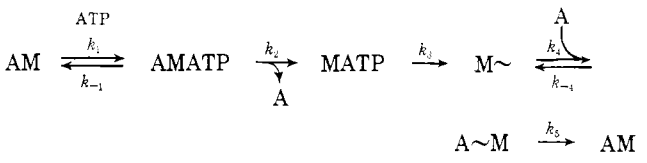
## Discussion

It is likely that all of the bound isotope is combined with myosin, because none of the other major proteins of the myofibril is capable of binding ATP in the concentration range studied (Bremel and Weber;[2] Weber, 1970). This assumption is consistent with the observed value for total bound

[1] The apparent decrease—as compared to normal myofibrils in the presence of calcium—of ATP binding subsequent to trypsin treatment may be an artifact of calculation. The protein content of the pellet was estimated exactly as for normal myofibrils, without separate controls for the solubilized actomyosin left in the supernatant. Since trypsin treatment increases solubilization of protein by ATP, the protein content of the pellet may have been significantly lower than the values used for calculation.

[2] R. D. Bremel and A. Weber, manuscript in preparation.

SCHEME I




isotope: as explained above, 2 μmoles/g of myofibrillar protein corresponds to 1 ATP per myosin head. More than 1 (Kiely and Martonosi, 1969) or nearly 2 nucleotide binding sites per molecule of myosin or heavy meromyosin has been previously found by others, when measuring the binding of ATP (Schliselfeld and Barany, 1968; Murphy and Morales, 1970) or ADP (Lowey and Luck, 1969; Morita, 1971).

It is likely that indeed 1 nucleotide is bound to each myosin "head" and not 2 to one and none to the other, because it can be shown that all active sites of heavy meromyosin as well as all S-1 molecules are capable of reacting with ATP. Each "head" of heavy meromyosin (Lowey, 1971) and all S-1 molecules (Young, 1967; Lowey, 1971) in the absence of ATP or other polyphosphates bind to actin and not one-half but nearly *all* such complexes with actin are dissociated by polyphosphates or ATP (Bremel and Beschorner, unpublished observations).

Furthermore, both active sites of a molecule seem to react with ATP in an identical manner. This statement is not in contradiction with the curvature of the Scatchard plot, because the number of sites in both classes is not equal, as it would have to be if each class represented one "head" on a myosin molecule. Instead 75% of all sites seem to bind nucleotide with the same $1/K_M$. This suggests that each "head" has the same kind of site, presumably a hydrolytic site, and that during interaction with actin and ATP the heads are probably independent of each other. The data do not support the existence of any "relaxing" or other special ATP binding site (Weber *et al.*, 1964; Levy and Ryan, 1967; Stewart and Levy, 1970).

These binding experiments do not distinguish bound ATP from ADP. Nevertheless binding of ADP from the medium must have been negligible because the concentration of free ADP was kept at such low levels. If myosin contains bound ADP it must be an intermediate in the series of reactions of substrate degradation before release of the product (Lymn and Taylor, 1970). The data by Marston and Tregear (1972) seem to suggest that a large fraction of the nucleotide bound to a relaxed actomyosin system is ADP and not ATP.

For further interpretation of the binding data one must know how many enzyme–substrate intermediates exist altogether, and how many contain bound isotope. Although precise information is lacking, one may make educated guesses, based on the kinetic studies by Eisenberg and Moos (1968, 1970) and Szentkiralyi and Oplatka (1969) and the rate constants measured by Lymn and Taylor (1970, 1971). Scheme I which also satisfies the requirements for shortening (*cf.* Huxley, 1957, 1969), is a slightly simplified[3] version of one proposed by Lymn and Taylor (1971) (AM = actomyosin, so-called rigor complex; M = myosin; A = actin; M~ = activated myosin with the terminal phosphate bond broken, but probably retaining phosphate (Tonomura and Kitagawa, 1960; Taylor *et al.*, 1970) as well as ADP (Lymn and Taylor, 1970, 1971); M~A = the high-energy actin–myosin complex, *i.e.*, the force-generating complex). Bound isotope is carried by several intermediates: AM–ATP, M–ATP, and M~. It has not been demonstrated whether M~A also contains bound ADP. If the estimation of myofibrillar myosin content by Hanson and Huxley (1957) is accurate, our data would be consistent with the assumption that M~A also contains bound ADP. The maximal number of bound nucleotide molecules equals the number of myosin heads, suggesting that intermediates free of bound nucleotide are not present in significant concentration. In our experiments we measured the sum of the concentrations of AM–ATP, M–ATP, M~, and possibly M~A, *i.e.*, with increasing concentrations of ATP the decrease in the concentration of AM, which depends on all of the rate constants and *A*.[4]

With this in mind we would like to discuss two points: (1) the effect of the removal of calcium on ATP binding and (2) the curvature of the Scatchard plot.

Calcium binding affects ATP binding although calcium and ATP are bound to different proteins: ATP is bound only to myosin (Bremel and Weber, 1972; Bremel and Weber[2]) and calcium to troponin (Ebashi *et al.*, 1968; Fuchs and Briggs, 1968). Furthermore, troponin is bound to actin filaments (Ohtsuki *et al.*, 1967; Ebashi *et al.*, 1969) and during relaxation acts only on actin (Weber and Bremel, 1972; Bremel and Weber[2]). The total number of binding sites is not altered by the removal of calcium. This observation does not support the concept of "relaxing sites" whose occupation is prevented by calcium (Steward and Ryan, 1970), a concept difficult to visualize anyway in view of the different localization of ATP and calcium. However, in the absence of calcium, ATP binding is saturated at lower ATP concentrations than in its presence, *i.e.*, the $K_M$ of ATP binding is decreased.

According to Scheme I, ATP binding may be described by the following equation (*cf.* Klotz and Hunston, 1971, see Appendix) ATP bound = $([ATP]K_1(1 + K_2)M_0)/(1 + [ATP]K_1(1 + K_2))$, whereby $1/(K_1(1 + K_2)) = K_M$; $K_1 = k_1/(k_{-1} + k_2)$ and $M_0$ = total number of myosin binding sites. $K_2$ contains the other rate constants given in Scheme I[5] and in the case of maximal activation of actin by calcium takes the following form: $K_2 = (k_2/k_3) + (k_2/k_4[A]) + (k_2/k_5) + (k_{-4}k_2/k_5k_4[A])$. In this equation the term [A] is assumed to be a constant, *i.e.*, the probability of collision between myosin and actin is independent of the ATP concentration. Consequently this equation does not account for the observed curvature of the Scatchard plot. The equation applies only to 75% of the sites that seem to obey only one set of constants whose value, however, changes with calcium removal. The curvature of the Scatchard plot will be discussed later, separately, for reasons that will become obvious then.

Taking into account first, the values of the constants measured by Lymn and Taylor (1971), second, $K_1(1 + K_2) = 5 \times 10^4$ for 75% of the sites (Figure 5), and third, $k_5$ = 100–200

[3] The main simplification consists of the omission of all back reactions for which we do not have constants. In addition, free myosin that has not reacted with ATP was ignored, since it is formed at such a slow rate as compared to AM.

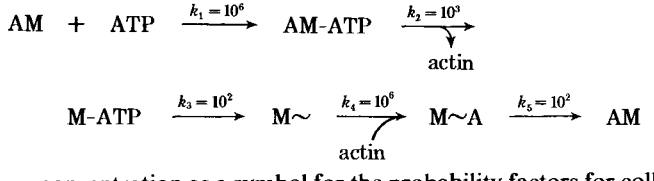
[4] The term *A* describes the probability of collision of myosin with actin at constant temperature; in a soluble system it would be represented by the concentration of free actin. For a myofibril with its actin molecules immobilized this parameter is not defined by the overall concentration of actin in the myofibril, but is an unknown factor.

[5] The reverse constants not indicated in the scheme have been neglected for more or less good reasons, such as lack of incorporation of $^{18}O$ into ATP, apparent complete dissociation of actomyosin by polyphosphates, and considerable decrease of $\Delta F$ in step 5.

TABLE I: Steady-State Concentrations of the Intermediates[a] as a Function of Increasing ATP Concentrations.

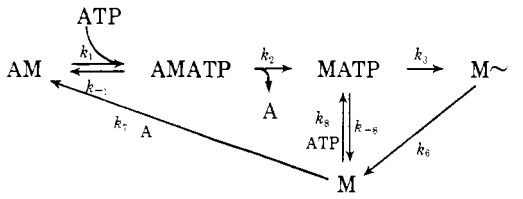
| ATP (μM) | $10^6$ ATP ($sec^{-1}$) | AM (μM) | AM–ATP (μM) | M–ATP (μM) | $10^6$ [Free Actin][b] ($sec^{-1}$) | M∼(μM) | M∼A (μM) | Total Bound Isotope, Sum of AM–ATP, M–ATP, M∼, and M∼A (μM) | Rate of ATP Hydrolysis (μM $sec^{-1}$) | Rate/Bound Nucleotide |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Calcium Saturated | | | | | |
| 0.5 | 0.5 | 190 | 0.095 | 0.95 | 10 | 9.5 | 0.95 | 11.5 | 95 | 9 |
| 1.0 | 1.0 | 184 | 0.184 | 1.84 | 14 | 13 | 1.84 | 17.8 | 184 | 10 |
| 5.0 | 5.0 | 160 | 0.8 | 8.0 | 32 | 25 | 8.00 | 42 | 800 | 20 |
| 10 | 10 | 140 | 1.4 | 14.0 | 46 | 30.4 | 14 | 60 | 1400 | 23 |
| 50 | 50 | 76 | 3.8 | 38 | 86 | 44 | 38 | 124 | 3800 | |
| $10^2$ | $10^2$ | 50 | 5.0 | 50 | 100 | 50 | 50 | 155 | 5000 | |
| $10^3$ | $10^3$ | 6.8 | 6.8 | 68 | 125 | 55 | 68 | 197 | 6800 | |
| $10^4$ | $10^4$ | 0.7 | 7.2 | 72 | 128 | 65 | 72 | 199 | 7200 | 36 |
| | | | | | No $Ca^{2+}$ | | | | | |
| | | | | | % Actin Inactivation | $10^6$ [Free Actin] ($sec^{-1}$)[c] | | | | |
| 0.5 | 0.5 | 178 | 0.089 | 0.89 | 80 | 4.2 | 21 | 0.89 | 22 | 89 | 4 |
| 1.0 | 1.0 | 168 | 0.168 | 1.68 | 81.3 | 5.6 | 30 | 1.68 | 34 | 168 | 5 |
| 2.0 | 2.0 | 142 | 0.29 | 2.9 | 89 | 5.95 | 48 | 2.84 | 54 | 284 | 5.3 |
| 5.0 | 5.0 | 110 | 0.55 | 5.5 | 92 | 6.75 | 81.5 | 5.5 | 90 | 550 | 6.1 |
| 10 | 10 | 77 | 0.77 | 7.7 | 94 | 6.9 | 112 | 7.7 | 120 | 770 | 6.4 |
| 50 | 50 | 12.2 | 0.65 | 6.2 | 97.9 | 3.8 | 162 | 6.2 | 175 | 620 | |
| $10^2$ | $10^2$ | 3.8 | 0.38 | 3.8 | 98.9 | 2.1 | 181 | 3.8 | 188 | 380 | |
| $10^3$ | $10^3$ | 0.4 | 0.4 | 3.8 | 99 | 2.0 | 190 | 3.8 | 199 | 380 | 1.9 |

[a] We used the following simplified reaction scheme with the following rate constants taken from Lymn and Taylor (1970, 1971), with the exception of the last two constants which were modified to fit our own kinetic data (Bremel *et al.*, 1972). (These constants are higher than those for systems containing purified actin, because of the elevated turnover rate of ATP by tropomyosin–troponin-containing systems.)




[b] All the concentration terms are arbitrary values, using the term concentration as a symbol for the probability factors for collisions in second-order reactions; free actin = total actin − AM − M∼A. [c] Free actin = (total actin − AM − M∼A) (100 − % inactivation)/100. The calculation was based on the following statements. (1) During steady-state conditions $10^6$[ATP][AM] = $10^3$[AM–ATP] = $10^2$[M–ATP] = $10^6$[actin][M∼] = $10^2$[M∼A]. (2) Total actin = total myosin heads = free actin + AM + AM–ATP + M∼A. (3) Total myosin heads = 200 μM = AM + AM–ATP + M–ATP + M∼ + M∼A (free myosin is too low to be significant). The numbers were obtained by iteration until all three equations were roughly satisfied.

SCHEME II




$sec^{-1}$ (Bremel *et al.*, 1972), one may put forward the following argument that ATP binding is essentially dependent on $K_2$. It cannot be determined solely by $K_1 = k_1/(k_{-1} + k_2)$, because $10^6/10^3$ ($k_2$ = at least $10^3$ according to Lymn and Taylor, 1971) does not give $5 \times 10^4$. In order to obtain that value—even if $k_{-1} \ll k_2$—the terms comprising $K_2$ must add up to a value of at least 50 for $K_1 + K_1K_2$ to give $5 \times 10^4$. With $k_2/k_3$ as well as $k_2/k_5 = 10$ ($k_3 = 100\ sec^{-1}$ according to Lymn and Taylor, 1971) and $k_{-4} \leqq k_5$,[6] the term $k_2/[A]k_4$ probably makes the major contribution to the reciprocal $K_M$ ($= K_1(1 + K_2)$).

How may these constants be affected by calcium binding to troponin? On first principles it may influence all reactions involving actin, either as free actin or complexed with myosin. In the latter case it is quite possible that a conformational change forced upon actin by troponin may induce a complementary conformational change in myosin bound to actin.

We know that during complete rest calcium removal has reduced step 4 to zero because during rest actin and myosin are completely dissociated. Consequently M~A has been eliminated from reaction Scheme I, and the hydrolysis of ATP occurs only through Scheme II.

Unfortunately, we did not see enough permissible simplifications to reduce this scheme sufficiently to obtain an analyzable $K_M$.

However, over the range of ATP concentrations used for measuring binding, relaxation was not complete; instead there was considerable actin-activated ATP hydrolysis (Figure 3), just as at these ATP concentrations shortening, syneresis, and tension development take place (Weber and Herz, 1963; Weber, 1970; Levy and Ryan, 1965; Endo, 1964; Reuben *et al.*, 1971; White, 1970). This behavior is not implicit in the reaction schemes nor is it elucidated by these binding studies. Observations that may offer an explanation for the persistence of actin–myosin interaction at low ATP after calcium removal are described elsewhere (Bremel and Weber, 1972). We will briefly summarize the conclusions. Since acto-S-1 systems behave just like actomyosin the biphasic response to ATP (Figure 3) is not based on interactions between ATP binding sites on the same myosin molecule. However, differences between ATP concentrations may be "sensed" by aggregates of S-1 molecules and one may assume that calcium removal inhibits only the interaction between actin and aggregates fully saturated with ATP. But there is no evidence for such aggregates although we cannot think of definite evidence against it. It is more likely that differences in the concentrations of ATP are sensed by the actin filaments through the amount of myosin bound as rigor complexes (= complex between actin and myosin that has not reacted with ATP). The binding constant of actin for myosin is greater than $10^7$. The lower the ATP concentration the less myosin contains bound nucleotide and therefore the greater the concentration of rigor complexes, *i.e.*, the higher the ratio actomyosin:actin. There is evidence that this ratio determines the extent of interaction between actin and myosin in the absence of calcium and that at a certain ratio (the precise value is not certain, it may be [AM~]/[A] = 0.3/1) all proteins of the actin filament are forced into a conformation similar to that induced by calcium binding to troponin. In support Bremel and Weber (1972) have demonstrated that the ratio actomyosin rigor complexes:total actin controls the calcium affinity of troponin.

As a result of these effects of AM (Scheme I) on actin conformation in the absence of calcium, ATP hydrolysis proceeded essentially through step 4 over most of the range of ATP concentrations used, and binding of nucleotide therefore is described by the same expression as in the presence of calcium. Nevertheless, at a given ATP concentration the flux through Scheme I is somewhat lower than in the presence of calcium (Figure 3) and considerably lower per bound nucleotide (Figure 6), indicating that the concentration of rigor complexes was never high enough to "turn on" actin maximally. Knowing that in the complete absence of rigor complexes calcium-free troponin reduces $k_4$[A] to zero it is reasonable to ascribe the reduction in the rate of hydrolysis to a reduction in the value of $k_4$[A]. Since $K_1(1 + K_2)$ seems to be largely determined by $k_2/k_4$[A][7] this reduction would account for the increase in nucleotide binding on calcium removal. It would follow that the rate per bound nucleotide was decreased because less of the nucleotide taken up was used to increase [M~A].

In conclusion, on the basis of all known constants of actin–myosin interaction, it is not unlikely that the $K_M$ for nucleotide binding is largely dependent on the rate of interaction of actin with activated myosin (M~) *i.e.*, the rate of formation of the force-generating links; therefore, a reduction of this rate by calcium removal should result in a lower value for $K_M$ of nucleotide binding.

The curvature of the Scatchard plot may indicate two classes of sites with different $K_M$ or negative cooperativity with a single class of sites. Because the low $K_M$ is associated with only 25 and not 50% of the sites, the two classes of sites cannot be represented by the two "heads" of myosin. They may belong to two different populations of myosin molecules. This possibility seems to be supported by the curved Scatchard plot of velocities of ATP hydrolysis by myosin alone (Lymn and Taylor, 1970) if it were not that Lymn and Taylor obtained their data by a very difficult technique which left some doubt in these authors' mind about their significance. Moreover two or more populations may easily be created as preparatory artefacts.

Even with only one population of myosin molecules with identical "heads," ATP binding may be governed by two $K_M$ values if—for steric reasons—some myosin molecules had little or no access to actin, because the $K_M$ for myosin alone is lower than that observed for actomyosin and that for actomyosin may well be governed by the step $k_4$[A] whereby accessibility = probability of collision = [A] (see above). In favor of this explanation is the observation that the $K_M$ for the first quarter of sites seems not to be affected by calcium removal.

[6] With $k_4 = 10^6$ (see Table I) and $k_5 = 10^2$ (Bremel *et al.*, 1972), $k_{-4}$ must be smaller than $k_5$ because the apparent $1/K_M$ = [A~M]/[A][M~] = $k_4/(k_{-4} + k_5)$ has a value between $10^5$ and $3 \times 10^3$ (Eisenberg and Moos, 1968; for troponin + tropomyosin containing actomyosin systems, R. D. Bremel *et al.*, 1972).

[7] The complete term is $(k_1k_2)/(k_{-1} + k_2)k_4$[A]).

Another possible way to explain the curvature of the Scatchard plot falls under the formalism of negative cooperativity. Negative cooperativity between myosin "heads" is not likely because the first abscissa intercept of the Scatchard plot occurs at 25% of the total sites. If binding of ATP to one myosin head inhibited binding to the second, or if there were a similar negative influence with respect to interaction with actin, the first slope should intersect at 1 or more sites out of 2, but never at less than 1. As pointed out above, the $K_M$ for ATP binding depends largely on interaction with actin, therefore the constancy of the $K_M$ for 75% of the sites suggests that each myosin head interacts independently and similarly with actin. Second there is a possible cooperativity of a more complex nature. An increase in $K_M$ caused by increasing ATP binding, *i.e.*, negative cooperativity, may occur if anywhere in the reaction scheme a rate-limiting rate constant increased as a result of ATP binding. We showed above that ATP binding may be governed by eq 1 and that on the basis of Taylor's constants in conjunction with our $K_M$ the dominant term is $K_2$ ($K_2 = (k_2/k_3) + (k_2/k_4[A]) + (k_2/k_5) + (k_{-4}k_2/k_5k_4[A])$), whose value depends largely on the value for $k_2/k_4[A]$. Therefore a decrease in this term would result in a decrease of the value of the $K_M$. With increasing ATP more and more actin is released from rigor complexes with myosin, the total amount of free actin is augmented, and it is possible that as a result the probability of collision of myosin with actin increases. It should be pointed out that it does not follow but is only possible: because we are dealing with an insoluble system it cannot be ruled out that each released actin molecule can interact only with the newly released myosin, so that $k_4[A]$ for each myosin molecule remains constant throughout the whole range of ATP concentrations. Which of the two possibilities holds must be eventually approached experimentally: if the curvature depended on an increase of $k_4[A]$ with increasing ATP or on its always having a different value for a parts of the myosin filaments, the curvature will not be present if the data are derived from a soluble system of actin and heavy meromyosin (HMM, the double-headed tryptic fragment of the myosin molecule). As long as the point cannot be decided yet, we have modeled a Scatchard plot, where in the presence of calcium the term [A] inceases proportionally with the total free actin released from actomyosin, and obtained, as indicated by Figure 7, a Scatchard plot that shows some of the features of the experimental data. As can be seen from Table I we arbitrarily chose a twelvefold increase of the term [A] although with twice as many actin molecules in a myofibril than myosin heads (Weber *et al.*, 1969) one may expect no more than a twofold increase. However, at this point so little is known about the probability of collision that we can defend our conditions by assuming that half the actin monomers are inaccessible at any one time and that the increase in [A] depends essentially on actin released from rigor complexes. All we want to point out is that such an increase in [A] is one of the possibilities to be considered. In the same spirit we modeled also in the absence of calcium, even more tentatively, because in this case there are—taking into account the evidence by Bremel and Weber (1972; see above)—two opposing effects of the release of actin from rigor complexes: (a) the increase in the probability of collision with increasing ATP is more and more offset by (b) the turning "off" of actin molecules with decreasing ratio of AM rigor complexes: total actin. However, we do not yet know what is the value of the ratio where actin molecules switch from the "on" to the "off" conformation. We may only assume that it is plausible that each unit of 7 monomers, 1 troponin, and 1 tropomyosin

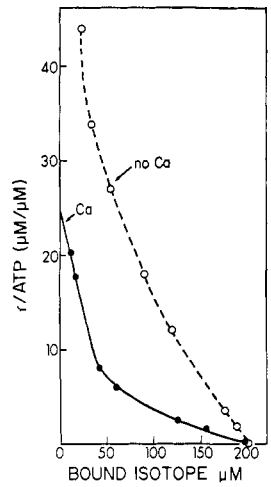



FIGURE 7: Scatchard plot of values for bound isotope calculated for the model described in the text. Numbers are of total bound isotope, Table I.

(Ebashi *et al.*, 1968; Bremel and Weber, 1972) is switched on and off separately and that with a statistical distribution of rigor complexes there will be a range of ATP concentrations where some units are on and some are off. In view of these uncertainties we just used the model in an attempt to indicate the general possibilities but we did not think it justified to spend the effort necessary to fit our data closely.

So far we have completely omitted from discussion the morphological state of the myofibril. Over the range of ATP concentrations used it changed from partially contracted at low ATP to either supercontracted in the presence of calcium (no structure visible under phase contrast) or to resting length in its absence. Nevertheless, these rather impressive differences may not much influence the binding of ATP. On increasing ATP the apparent $K_M$ always increased, although in one case the myofibrils were supershortened and in the other at resting length.

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## Appendix

The calculation for $1/K_M$ of ATP binding is based on the fact that during steady-state hydrolysis each step of reaction Scheme I proceeds at the same rate. Back reactions are neglected except for steps 1 and 4.

Abbreviations used in the following equations are: $M_0$, total ATP binding sites of myosin; MATP, binding sites on free myosin; [A], probability of collisions of myosin with free actin; M~, myosin that has reacted with ATP, very likely myosin ADP·P (Lymn and Taylor, 1970); A~M force-generating complex; AM, actomyosin rigor complex.

$$k_1[AM][ATP] - k_{-1}[AMATP] = k_2[AMATP] = k_3[MATP] = k_4[A][M\sim] - k_{-4}[A\sim M] = k_5[A\sim M] \quad (1)$$

$$\frac{k_1}{k_{-1} + k_2} = K_1 \tag{2}$$

$$\text{Nucleotide bound} = [\text{AM}][\text{ATP}]K_1a \tag{3}$$

In eq 3 and 4 $a = 1 + (k_2/k_3) + (k_2/k_4[\text{A}]) + (k_2/k_5) + (k_2k_{-4}/k_4[\text{A}]k_5$; $a$ must have a value of at least 50; $[\text{AM}] = M_0 - [\text{AM}][\text{ATP}]K_1a$.

$$[\text{AM}] = \frac{M_0}{1 + [\text{ATP}]K_1a} \tag{4}$$

$$\text{Nucleotide bound} = \frac{M_0[\text{ATP}]K_1(1 + K_2)}{1 + [\text{ATP}]K_1(1 + K_2)} \tag{5}$$

$$K_2 = \frac{k_2}{k_3} + \frac{k_2}{k_4[\text{A}]} + \frac{k_2}{k_5} + \frac{k_2k_{-4}}{k_4[\text{A}]k_5} \tag{6}$$

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