k_{-6} 2 sec^{-1}, k_7 125 sec^{-1}, and k_3/k_{-3} 0.084 mM^{-1}; the constant for Ca-dissociation from troponin was evaluated with $K_T = 3.8 \cdot 10^{-4}$ mM and the allosteric constant L' with 10 mM^{-1}. The mean percentage deviation between the model predictions and the ATPase and binding studies shown in Fig. 1 A, C and D was 14.86%. To fit the Ca-binding data in Fig. 1 B (in absence of NEM) an equilibrium constant of $2 \cdot 10^5$ mM^{-1} for the binding of Ca^{2+} to myosin had to be additionally

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To establish a biochemical basis for the low-temperature thermo-chemical and mechanical studies on frog sartorius (1-3), the steady-state kinetics of frog sartorius myofibrillar ATPase have been studied at 1.5 °C. Variations in the level of ATP and Ca^{2+} ions provide some detailed information on the relationship between actomyosin ATPase activity and ligand binding.

Material and Methods

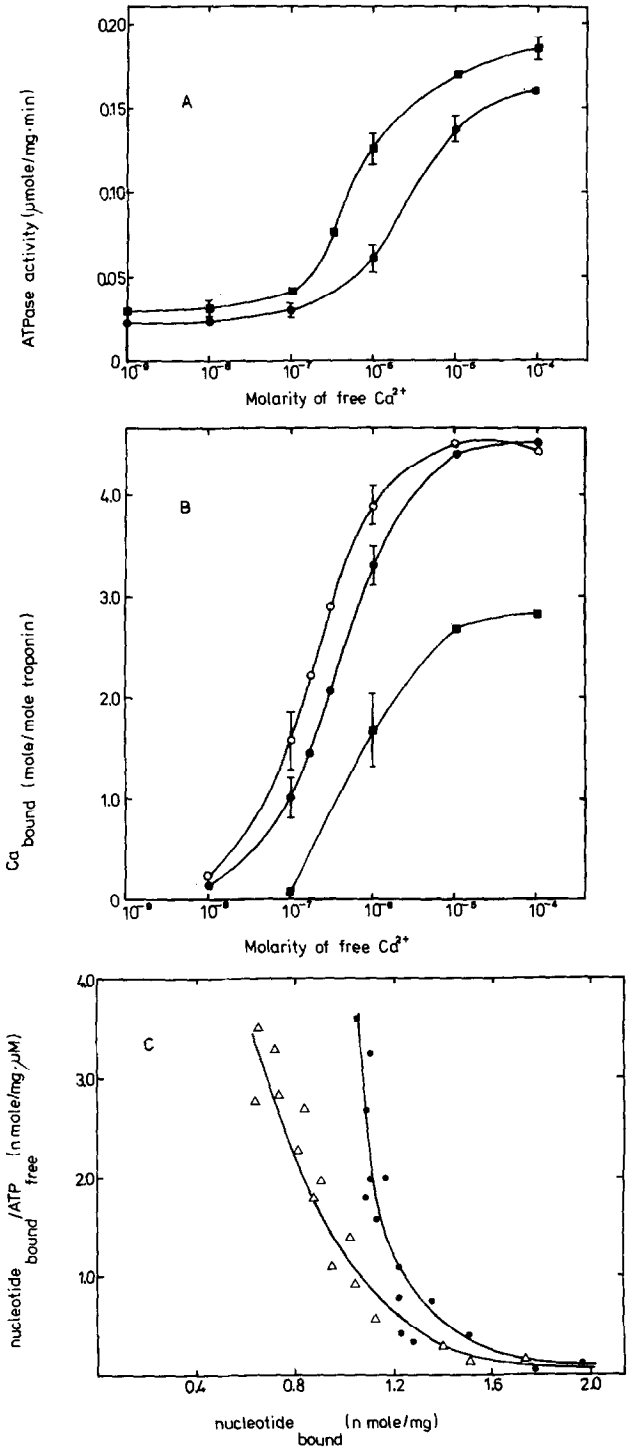
Myofibrils were prepared from frog sartorius by the method of Weber, Herz and Reiss (9). The relative content of the myofibrillar protein components (total of 75-76 mg protein/gram wet weight of muscle) was estimated by sodium dodecyl sulfate gel electrophoresis (10), giving values per gram muscle equal to 0.085 μmole myosin, 0.36 μmole actin, 0.048 μmole troponin and 0.051 μmole tropomyosin. Thus, similar as in rabbit myofibrils the ratio of troponin to actin molecules is about 1:7 (8) with one tropomyosin molecule covering seven actin monomers (11). The finding that frog troponin cross-reacted with an antibody prepared against rabbit troponin (12) suggests a closely related structure.

ATPase activity as well as nucleotide and Ca^{2+} binding was determined at 1.5°C in a medium with the following composition: 4 mg/ml myofibrillar protein, 10 μM - 2 mM MgATP, 4 mM Ca-EGTA/EGTA buffer to stabilize the concentration of free Ca^{2+} ions (5), 10 mM imidazole (pH 7.0), 4 mM creatine phosphate, 0.5 mg/ml creatine kinase, with the ionic strength adjusted to 0.11 with KCl. The experimental conditions followed closely those used for rabbit myofibrils (7,8). The myofibrils were quickly separated from the medium by filtration through Millipore filters mounted on test tubes with side arms connected to a vacuum line. For the binding studies the medium contained [8-^{14}C] ATP (Schwarz, 10.1 mC/mmole) or ^{45}Ca-EGTA (Amersham, 20 mC/gm Ca). As an indicator of solvent space in the pellet 5 mM ^{3}H-glucose was used (8). The concentration of bound ^{14}C-labelled nucleotide and ^{45}Ca was calculated from the radioactivity in the pellet in excess of that in the incubating solution. The isotopes were extracted with

5% TCA and counted in an I.D.L. two-channel Tritomat 6020. Internal standards, including also [U-^{14}C]ADP, were recovered by 94-97%. The ATPase activity was determined from the amount of creatine liberated (3) during the 1 min incubation period.

Results

Similar to the findings on rabbit myofibrils (5-9) increasing the concentrations of Ca^{2+} ions from 10^{-9} to 10^{-4} M leads to a progessive activation of the myofibrillar ATPase from frog sartorius muscle (Fig. 1A). This activation was less effective at the higher MgATP levels. Making the justified assumption that Ca^{2+} binding to troponin exerts a regulatory effect similar to that in the rabbit system (6), ligand binding to frog myofibrils was measured using ^{45}Ca. To facilitate a comparison with rabbit myofibrils (8) MgATP concentrations of 10 μM and 2 mM were chosen. If the Ca-binding was expressed in terms of the troponin content is could be shown that maximally 4.5 mole ^{45}Ca became bound per mole of troponin in presence of 10^{-5} M Ca^{2+} (Fig. 1B). However, at the lower MgATP concentration there was an apparent affinity increase for Ca^{2+}, which has previously been observed for rabbit myofibrils (8). As the Ca-binding to troponin is unaffected by the level of ATP (cf (4)).and the myosin also possesses a Ca-binding site (13) an attempt was made to distinguish between Ca-binding to troponin and myosin. Ca-binding to myosin and the relaxability of acto-myosin have been reported to be particularly sensitive to low levels of N-ethyl maleimide or NEM (14). When the myofibrils were incubated with 2 mμM NEM/mg myofibrillar protein the modifying effect of MgATP on Ca-binding was no longer detectable (Fig. 1B). Further, no ^{45}Ca became bound at Ca^{2+} levels lower than 10^{-7} M with the total amount of ^{45}Ca bound reduced by about one third even at 10^{-5} and 10^{-4} M Ca^{2+}. It seems therefore likely that Ca-binding at the very low Ca^{2+} levels reflects the binding of one ^{45}Ca molecule per myosin cross-bridge. This would certainly be consistent with there being one Ca-binding site on the myosin ligth chains which has a binding constant of $102 \cdot 10^{6} M^{-1}$ (13). If the Ca-binding in presence of NEM reflects indeed the binding to the troponin component then


A
ATPase activity (μmole/mg·min)
0.20
0.15
0.10
0.05
0
Molarity of free Ca2+
B
Ca bound (mole/mole troponin)
4.0
3.0
2.0
1.0
0
Molarity of free Ca2+
C
nucleotide bound/ATP free (n mole/mg·μM)
4.0
3.0
2.0
1.0
0.4
0.8
1.2
1.6
2.0
nucleotide bound (n mole/mg)

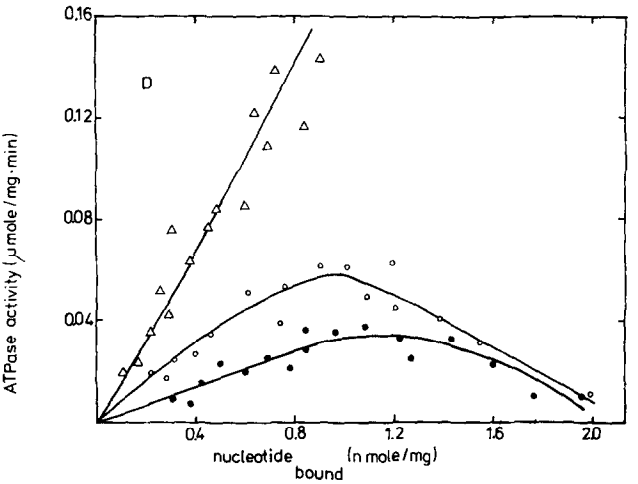


Figure 1. Steady-state kinetics of myofibrillar ATPase from frog sartorius muscle.
A. The influence of the levels of ATP and Ca^{2+} ions on ATPase activity. The MgATP concentrations were 0.5 mM (■) and 5 mM (•).
B. Effect of ATP on Ca-binding. ^{45}Ca-binding was measured in presence of 10 μM (○) and 2 mM MgATP (•). When 2 m μM N-ethyl maleimide/mg myofibrillar protein was additionally present (■) the results obtained at 10 μM and 2 mM MgATP became indistinguishable in view of the considerable scatter. In Fig. 1 A & B characteristic error bars are given (mean ± S.E., 15 experiments).
C. Scatchard plot of nucleotide binding. Nucleotide binding was related to the free MgATP concentration at 10^{-9} M Ca^{2+} (•) and 10^{-4} M Ca^{2+} (△).
D. Myofibrillar ATPase activity as a function of nucleotide binding. The results were obtained in presence of 10^{-9} M (•), 10^{-7} M (○) and 10^{-4} M Ca^{2+} (△). In Fig. 1 C & D the points refer to individual experiments.

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there must be three binding sites per troponin molecule as can also be deduced from studies on rabbit treponin (6, 12, 15).
In view of the marked effects of the MgATP level on both Ca^{2+}-stimulated ATPase activity and Ca-binding to myofibrils (Fig. 1 A & B) nucleotide binding in presence of characteristic Ca^{2+} levels was recorded (Fig. 1 C & D). The Scatchard plot (16) of Fig. 1 C shows the binding of total nucleotide in relation to the free ATP level in the medium both in the virtual absence of Ca^{2+} and at maximally activating Ca^{2+} levels. It is important to note that removal of Ca^{2+} does not increase the number of nucleotide binding sites as has also been

reported for rabbit myofibrils (8), although the fraction of bound ADP is likely to be higher at the very low Ca^{2+} (17). In contrast to the rabbit system where Ca^{2+} was found to convert a low-affinity ATP-binding constant to a high-affinity one (7), the low-affinity ATP-binding constant in frog myofibrils is not affected by the Ca^{2+} level as the slope at higher amounts of nucleotide bound remaine essentially the same (Fig. 1 C).

The relation between ATPase activity and nucleotide binding (Fig. 1 D) exhibits the same tendency as observed for rabbit myofibrils (7). At maximally activating Ca^{2+} levels less nucleotide is bound. The lower activity of the ATPase at 10^{-7} or 10^{-9} M Ca^{2+} (see also Fig. 1 A) is associated with an increase in nucleotide binding. This finding is certainly consistent with the concept of negative cooperativity between the two myosin heads proposed to explain the effect of MgATP on ADP release in the pre-steady state (18). The attempt was therefore made to fit the present set of data to the kinetic schema suggested for rabbit actomyosin (18).

Evaluation of the results in terms of a kinetic model

The kinetic scheme in Fig. 2 which was derived from pre-steady state studies on rabbit myosin and acto-myosin (18) makes several model assumptions. In the nucleotide-free acto-myosin complex (state 1) both heads on the myosin cross-bridge (or S1 subunits) have the same affinity for ATP. Upon binding of ATP to one of the S1 subunits the myosin cross-bridge detaches from the actin, the state 2 being formed with the over-all rate constant k_1. However, due to negative cooperativity between the two myosin heads the affinity for ATP becomes reduced in the second S1 subunit. The ATP molecule bound to the first S1 subunit is split extremely fast (k_2). While one of the products, the inorganic phosphate, is liberated from the myosin-product complex (state 3) with a high rate constant, k_5, ADP is only released at a significantly fast rate after actin interacts again with the myosin-ADP complex (compare Lymn and Taylor (23)), the association and dissociation constants of the acto-myosin·ADP complex being k_6 and

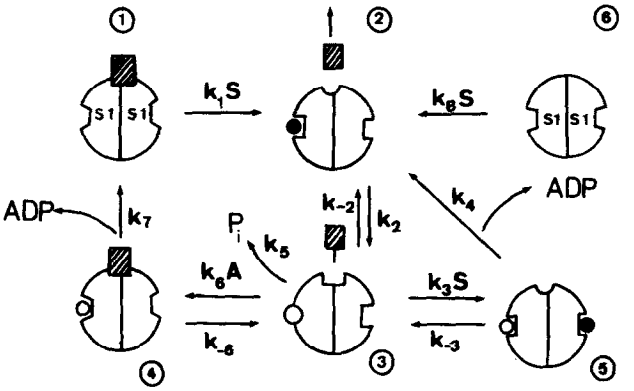

Figure 2. Kinetic scheme for myofibrillar ATPase. The two heads on the myosin cross-bridge have been indicated by the two S1 subunits. The actin is denoted by the shaded squares, while the binding of ATP to states 2 and 5 is indicated by the shaded circles and that of ADP to states 3, 4 and 5 by the open circles.

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k_{-6}. As ADP is now able to dissociate readily (k_7) state 1 is re-attained.

While the second S1 subunit in state 3 has a lower affinity for ATP the extent of ATP binding will become quite substantial at millimolar ATP levels, so that state 5 is formed with the kinetic constant k_3. This reaction will be reversible, hence only the ratio of k_3/k_{-3} will be of importance. As the ATP on the second S1 subunit is not hydrolyzed and, further, prevents the interaction with actin the ADP still bound to the first S1 can only dissociate with the very slow rate constant k_4 (18). In view of the fact that the experiments have been carried out in presence of an ADP-rephosphorylating system the ADP dissociation steps can be considered as irreversible.

In the previous formulation of the kinetic scheme (18) the assumption was inherent that all actin sites were in fact available for interaction with the myosin, as is true for maximally activating conditions. However, to account for the activating effect of Ca^{2+} ions a reaction step needs to be additionally introduced at the level of the troponin which governs the "turning on" of the actin molecules. The regulatory unit of one troponin-tropomyosin covering seven actin monomers (11)

is known to operate through a conformational change induced in the troponin as the result of Ca^{2+} binding which leads to a gradual displacement of the tropomyosin masking the actin monomers (19,20). Additionally, it was found by Bremel and Weber (8) and confirmed by Haselgrove (20) that nucleotide-free myosin cross-bridges by attaching stably to the actin equally lead to a displacement of the tropomyosin and hence "turn on" neighbouring actin molecules. If the MgATP concentration through the relative fraction of such rigor complexes (state 1 in Fig. 2) would affect the transition between two states of the troponin differing markedly in their affinity for Ca^{2+} as postulated by Bremel and Weber (8) it becomes possible to apply the allosteric model of Monod, Wyman and Changeux (21).

The equilibrium constant L between the two-troponin states would be the product of the ATP concentration , S, and an allosteric constant L'. For the case that Ca^{2+} only binds to one of the two troponin states ($Ca/K_T = \alpha$) the actin concentration, A, available for interaction with the myosin, M, can be predicted by the equation

$$A = \frac{(1+\alpha)^{n-1}}{L' \cdot S + (1+\alpha)^n} \qquad (1),$$

where the number of Ca-binding sites on the troponin is n = 3 (6,12,15).

As the splitting of ATP (k_2) and the liberation of P_i (k_5) are very fast steps and the concentration of free myosin (state 6) will be negligible under the experimental conditions, the kinetic equations necessary to describe the steady ATPase can be reduced from those inherent in the scheme of Fig. 2. The velocity of the myofibrillar ATPase follows from

$$v = \frac{M\, k_7\, k_1 S\, k_6 A\, k_4}{k_7\, k_6 A + (k_7 + k_{-6}) k_1 S + k_1 S\, k_6 A + (k_3 S / k_{-3})(k_7 + k_{-6}) k_1 S} \qquad (2).$$

The amount of nucleotide bound, i.e. the total concentration of ATP plus ADP, can be calculated from the relative fraction of the various states predicted by the kinetic constants. Using equ. (2) it becomes possible to predict the results in Fig. 1 A, C and D with the following set of parameters evaluated by the optimal search strategy of Reich et al. (22): k_4 0.05 sec^{-1}, k_1 73 $mM^{-1}sec^{-1}$, k_6 680 $mM^{-1}sec^{-1}$,

k_{-6} 2 sec^{-1}, k_7 125 sec^{-1}, and k_3/k_{-3} 0.084 mM^{-1}; the constant for Ca-dissociation from troponin was evaluated with $K_T = 3.8 \cdot 10^{-4}$ mM and the allosteric constant L' with 10 mM^{-1}. The mean percentage deviation between the model predictions and the ATPase and binding studies shown in Fig. 1 A, C and D was 14.86%. To fit the Ca-binding data in Fig. 1 B (in absence of NEM) an equilibrium constant of $2 \cdot 10^5$ mM^{-1} for the binding of Ca^{2+} to myosin had to be additionally

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