FLUORIMETRIC STUDIES ON THE INFLUENCE OF METAL IONS AND CHELATORS ON THE INTERACTION BETWEEN MYOSIN AND ATP

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1. Introduction

Although it is well known that magnesium and ATP are necessary for the working of muscle and that this Mg-ATPase is located in the head portions of the myosin molecule, little direct evidence has been presented on the nature of the enzyme–substrate complex (for recent reviews see [1 and 2]). This report is concerned with fluorimetric studies on the interaction between myosin (or its subfragments heavy meromyosin (HMM) and HMM-subfragment 1 (HMM-S1)) and its substrate ATP in the presence or absence of metal cations. The change of the intrinsic tryptophan fluorescence of myosin upon interaction with substrate is a sensitive probe for local conformational changes of the enzyme and moreover makes accurate measurements of enzymatic activity possible, thus providing two characteristic features of the ATPase mechanism. From the experiments we draw the following main conclusions*:

i) Mg^{2+}-ATP and not ATP is hydrolysed and during the reaction forms an intermediate complex of the form EMS such that the enzyme E and the substrate S protect the metal ion M from chelators in the solvent; free ATP inhibits the Mg^{2+}-ATPase by binding at the active site without hydrolysis.

ii) The rate of the back dissociation of the enzyme–substrate complex is very slow ($k_{-1} < 0.02$ sec^{-1}) but increases if Mg^{2+} is removed or replaced by Na^{+} ($k_{-1} \cong 0.2$ sec^{-1}).

iii) There is no experimental evidence for a cooperativity between the two catalytic centers of myosin and HMM; both active sites seem to function independently.

* Details on the effect of various metal cations and substrate analogs will be reported elsewhere [3].

2. Materials and methods

Rabbit myosin, HMM, and HMM-S1 were prepared by the method of Lowey et al. [4] with slight modifications. Standard conditions for fluorimetric measurements were 50 mM Tris-HCl buffer pH 7.5 for HMM and HMM-S1, with the addition of 0.5 M KCl when myosin was used. In the former case the total concentration of mono- or divalent metal cations is estimated to be less than 0.1 mM; the case where residual divalent cations are removed by chelators will be referred to as "no ions" (this is justified because monovalent cations – in contrast to divalent cations – become effective only at concentrations exceeding about 10 mM). Protein concentrations were between 2 and 6 μM calculated on the basis of $A^{1\%}_{280,1\,cm} = 6.0$, 6.4 and 8.0 and molecular weights of 460 000, 365 000, and 115 000 for myosin, HMM, and HMM-S1, respectively. Fluorescence measurements were made on a Hitachi MPF3A spectrofluorimeter with a temperature controlled sample holder set at 20 ± 0.5°C. Excitation and emission wavelengths were 300 and 336 nm with band widths of 3 and 6 nm. Fluorescence levels and changes are quoted in percent of the initial level of the protein in the standard solution (= 100%) with errors of about 1%. Errors in rate constant mea-

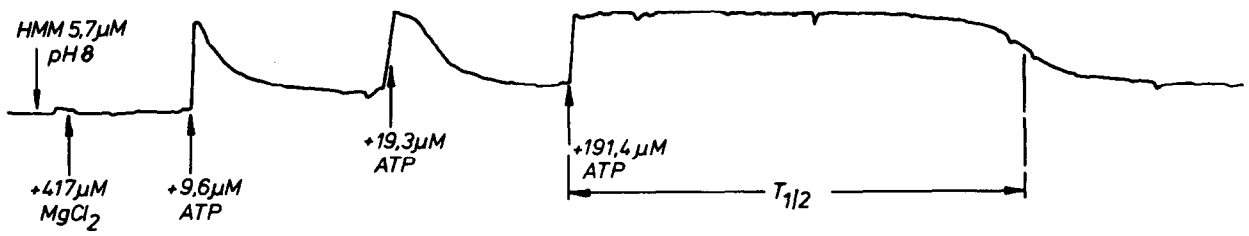

Fig. 1. Typical experiment to determine decay constants and enzymatic activity. Conditions 1.2 ml HMM 5.7 μM (11.4 μM sites), 50 mM glycine-HCl buffer pH 8.8 (selected for illustration because of the high ATPase activity), no salt added, 20°C. Decay constants of the one and two shot experiments are 3.5 and 3.6 min^{-1}, activity v_{max} = 7.7 min^{-1} ≈ 2 k_{decay}. Linear dilution correction $F_{corr} = F_{obs} \cdot V_{total}/1.2$ ml.

surements are 10 to 20%. Potassium salts of ATP and β, γ-imido-ATP (APPNP) were obtained from Pabst Laboratories and Boehringer, Mannheim. All cations were added as chloride salts. Analytical grade reagents were used throughout. Since we find no differences in the enzymatic behavior between myosin and its subfragments except those which can be accounted for by the different salt concentrations, most experiments are described for the case of HMM unless noted otherwise.

Enzymatic activities were measured fluorimetrically using Chance's equation as described by Morita [5],

$$v_{max} = s_0/(e_0 T_{1/2})$$

where s_0 and e_0 are initial concentrations of substrate and enzyme, $T_{1/2}$ is the time at which the fluorescence drops midway between its maximal and final levels (cf. fig. 1). The activities thus obtained agreed well with pH-stat measurements (Radiometer Titrigraph TTT1) and made the use of the latter unnecessary.

3. Results and discussion

As a basis of discussion we use the reaction scheme proposed by Lymn and Taylor [6] and extended by Bagshaw et al. [7]:

$$My_1 + ATP \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} My_2 \cdot ATP \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} My_3 \cdot ADP \cdot P_i \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} My_4$$

$$\times ADP \cdot P_i \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}} My_5 \cdot ADP + P_i \underset{k_{-5}}{\overset{k_5}{\rightleftharpoons}} My_1 + ADP + P_i$$

Here subscripts are used to denote possibly different conformational states of the enzyme (My = myosin, HMM, or HMM-S1). As shown by Werber et al. [8] the intrinsic tryptophan fluorescence of myosin is enhanced upon interaction with various substrates. The experiments to be described are based on the observations [9] that

i) the maximal fluorescence enhancement (ΔF_{max} = 13, 18, and 22% for myosin, HMM, and HMM-S1) is induced by substrates whose hydrolysis is rate limited by step k_3, i.e. those which show the "early burst" phenomenon [10]. Since the steady state complex is predominantly $My_3 \cdot ADP \cdot P_i$ in this case, it is concluded that ΔF_{max} is characteristic of the My_3-conformation. Examples are Mg^{2+}-ATP and Ca^{2+}-ATP [6].

ii) In contrast, if substrates are chosen for which step k_2 is rate limiting or zero (binding without hydrolysis), the fluorescence change reaches only 1/3 to 2/3 of ΔF_{max} which is taken to be the fluorescence of the My_2-conformation. Examples are ATPγS [7, 11], α,β-methylen-ATP [12], K^+-ATP [6] and non-hydrolysable substrate analogs such as Na^+-ATP, ADP, and APPNP [13].

iii) EDTA (ethylene diamine tetraacetic acid) chelates divalent metal cations and can thus be used to suppress the fluorescence of any divalent cation mediated ATPase (rate limited by k_3), and to make visible the influence on the ATPase due to monovalent ions which are added to the solution.

3.1. *Experiments with EDTA*

When residual divalent metal cations in the enzyme preparation are chelated by EDTA the fluorescence rises by 3 to 6% immediately, depending on preparation. The effect is reversible by excess magnesium.

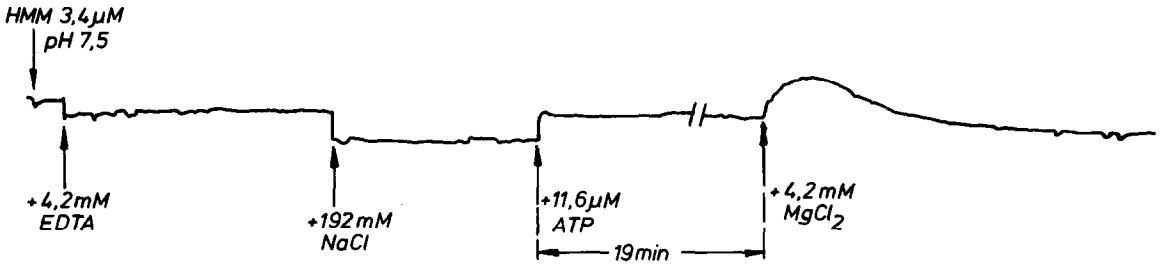

Fig. 2. Influence of Na^+ on the ATPase. Conditions 1 ml HMM 3.4 μM, 50 mM Tris-HCl buffer pH 7.5. EDTA and NaCl cause enhancements of 3.1 and 2.5% (after dilution correction), rising to 7.2% after addition of ATP. No ATP is hydrolysed. The fluorescence rise after Mg^{2+} addition is at a rate of 8.5 min^{-1}.

EGTA, on the other hand, only produces a slight change (never more than 2%) indicating that the quenching of the fluorescence is mainly caused by Mg^{2+} and not by Ca^{2+} ions.

Subsequent addition of ATP increases the fluorescence by 3 to 6% above the EDTA level depending on the nature and concentration of the monovalent cations added to the solution (fig. 2). Whereas Li^+, K^+, Rb^+, Cs^+, and NH_4^+ support ATP hydrolysis, there is no activity but binding in the case of "no ions" and with Na^+ [14]. The fluorescence enhancement is clearly lower than ΔF_{max} which can only be reached by compensating the EDTA effect with excess Mg^{2+}.

On the other hand, if Mg^{2+} and ATP are added first so that the high fluorescence of the steady state complex (ΔF_{max}) is reached, excess EDTA causes the rapid rise by 3–6% typical of the removal of quenching ions followed by a slow fluorescence drop (fig. 3). Its rate constant k_{decay} can be correlated with the steady state velocity v_{max} of the Mg^{2+}-ATPase in terms of moles substrate/moles enzyme/min:
$k_{decay} \cong 0.5\, v_{max}$ for myosin and HMM,
$k_{decay} \cong v_{max}$ for HMM-S1.

These observations suggest the following conclusions:

i) The quenching effect of Mg^{2+} ions and its reversibility by EDTA is at least partially independent of the conformation of the enzyme. This probably means that those Mg^{2+} ions which mediate the Mg^{2+}-ATPase are not identical with those which quench the fluorescence.

ii) The conformation My_3 is not attained unless the enzyme, Mg^{2+}, and ATP form the $My_3 \cdot ADP \cdot P_i$ complex of the type EMS so that the metal ion M is protected from chelation by EDTA and becomes available only after the breakdown of the complex.

iii) Free ATP (i.e. ATP not complexed with metal cations) and Na^+-ATP are bound but not hydrolysed by the enzyme, with dissociation constants around 1 μM. The rise to the maximal fluorescence upon addition of excess Mg^{2+} occurs at a rate of 0.2 sec^{-1}. This rate does not depend on the concentration of ATP, Na^+, and Mg^{2+} as long as Mg^{2+}-ATP is sufficient to saturate the enzyme and therefore can be taken as the first order dissociation rate (k_{-1}) of ATP and Na^+-

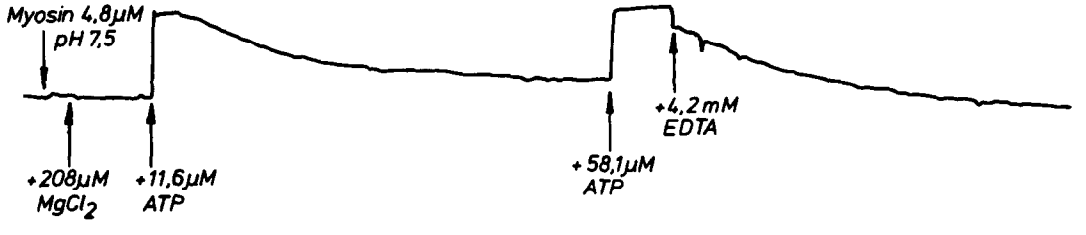

Fig. 3. EDTA-induced fluorescence decay. Conditions 1.2 ml myosin 4.8 μM, 50 mM Tris-HCl buffer pH 7.5, 0.5 M KCl. Both decay constants are 0.9 min^{-1}, the ATPase activity is 1.8 min^{-1}.

ATP*. This suggests that ATP or Na^+-ATP have to leave the enzyme before Mg^{2+}-ATP can bind so that the association mechanism is of the form E + MS → EMS whereas the type ES + M → EMS seems not possible.

iv) Assuming that chelation of Mg^{2+} is fast enough to prevent any rebinding of Mg^{2+}-ATP or Mg^{2+}-ADP after discharge from the enzyme, the breakdown of the high fluorescence of the complex $My_3 \cdot ADP \cdot P_i$ occurs at a rate [15]

$$k_{decay} = k_3 \cdot \frac{k_2}{k_2 + k_{-2}} + k_{-1} \cdot \frac{k_{-2}}{k_2 + k_{-2}}$$

whereas the enzymatic velocity is essentially determined by the rate limiting step k_3. Since $k_{+2} \approx 10\ k_{-2}$ [15], about 10% of the enzyme is in the state $My_2 \cdot ATP$, and therefore if k_{-1} were large, k_{decay} would have to exceed k_3 measurably. However, we find that $k_{decay} \approx k_3$ ($= v_{max}$ for HMM-S1) which means that k_{-1} must be small for Mg^{2+}-ATP as a substrate. Considering the experimental error the value of k_3 ($\approx 0.02\ sec^{-1}$) is an upper limit for k_{-1}, in good agreement with the value obtained by a different method [15]. The dissociation constant is then calculated to be $K_d = k_{-1}/k_{+1} < 2 \times 10^{-8}$ M.

v) The fact that $v_{max} \cong 2\, k_{decay} \cong 2\, k_3$ in the case of myosin and HMM can be taken to indicate that a measurable cooperativity between the two enzymatic centers of the molecule which would amount to significantly less than two effective centers does not exist. This latter possibility was suggested by binding studies (including our own) which show less than two substrate binding sites per myosin molecule (e.g. [16, 17]). At any rate both a large k_{-1} and the above cooperativity would have the effect of increasing k_{decay} over $0.5\, v_{max}$ which is not observed.

The fluorescence decay caused by EDTA is a good example for the usefulness of fluorimetric activity measurements as compared to other methods: In the case of myosin which was dissolved in 0.5 M KCl the chelation of Mg^{2+} is accompanied by a strong activation of the ATPase (up to 300-fold) whereas HMM and HMM-S1 are soluble without salt so that the ATPase activity drops to zero without Mg^{2+}. In both cases, however, the same slow decay of the Mg^{2+}-ATPase can be observed during which only one molecule of ATP is split per active site, the remainder being hydrolysed by a different mechanism or not at all.

3.2. *The decay of the intermediate enzyme–substrate complex*

If ATP is added to the enzyme in the presence of Mg^{2+} such that all Mg^{2+}-ATP is firmly bound, the decay of the ES complex (from ΔF_{max} to about 0.4 ΔF_{max}) is observed essentially without competitive interference from products†. The results substantiate the observations made with EDTA, i.e. we have $k_{decay} \approx k_3 \cong v_{max}$ for HMM-S1 and $k_{decay} \approx 0.5\ v_{max}$ for myosin and HMM, again showing that neither cooperativity nor back dissociation via k_{-2} and k_{-1} play a measureable role (fig. 1).

3.3. *The formation of the enzyme–substrate complex*

Having excluded an association process of the type ES + M → EMS, the two remaining possibilities are EM + S → EMS and E + MS → EMS. The former is suggested intuitively from the reported strong binding of Mg^{2+} ions to the enzyme [17]. However, the experiments indicate that the latter process is dominant for the following reasons:

i) When increasing amounts of ATP are added to the enzyme containing residual Mg^{2+} ions, the fluorescence enhancement increases and becomes nearly maximal at ATP concentrations above 0.1 mM; at the same time the apparent association rate slows down noticeably at concentrations of free ATP above 1 mM. This behavior excludes the process EM + S → EMS which would imply that the apparent association rate be proportional to (S), but it is consistent with the assumption that initially increasing (ATP) increases (Mg^{2+}-ATP) binding quickly and tightly to the enzyme whereas at high ATP concentrations competition from free ATP (whose dissociation constant is at

* With $K_d \approx 1\,\mu M$ and $k_{-1} \approx 0.2\ sec^{-1}$ we calculate $k_{+1} \approx 10^5\ M^{-1}\ sec^{-1}$ for Na^+-ATP which is ten times slower than k_{+1} of Mg^{2+}-ATP [6] so that association of Mg^{2+}-ATP cannot be rate limiting at the concentrations used.

† This statement is valid if quick transients arising from the equilibration between enzyme and products are neglected. We are grateful to Dr. Trentham for a discussion of the rate constants

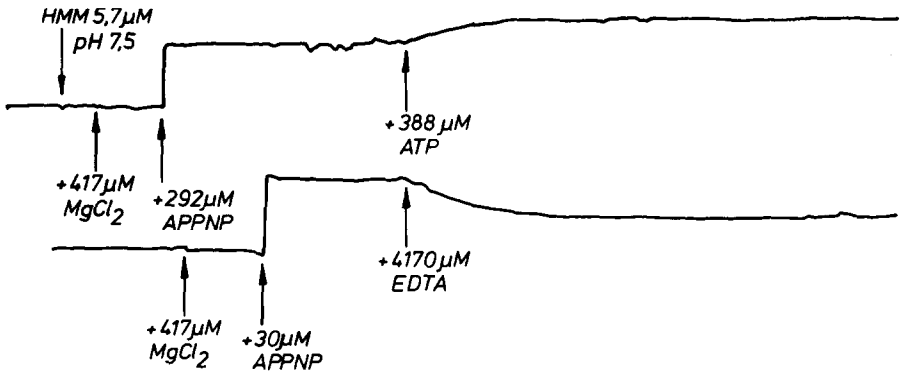

Fig. 4. Dissociation of Mg^{2+}-APPNP from HMM. Conditions 1.2 ml HMM 5.7 μM, 50 mM Tris-HCl buffer pH 7.5. Upper curve: APPNP binds in presence of Mg^{2+} and is replaced by subsequent ATP at a rate of 0.05 sec^{-1}. Lower curve: Dissociation of bound Mg^{2+}-APPNP after addition of excess EDTA at a rate of 0.05 sec^{-1}.

least two orders of magnitude larger than that of Mg^{2+}-ATP) becomes noticeable.

ii) The substrate analog APPNP induces a change of $\Delta F = 2/3\ \Delta F_{max}$ in the presence of Mg^{2+}. If EDTA is added the fluorescence drops at a rate of 0.05 sec^{-1} showing first that Mg^{2+} is protected in the My_2–APPNP conformation, and second that the rate of dissociation of Mg^{2+}-APPNP is $k_{-1} = 0.05\ sec^{-1}$ which agrees with the value published previously [7]. If ATP is added instead of EDTA, the fluorescence level rises at about the same rate as Mg^{2+}-APPNP is displaced from the enzyme by Mg^{2+}-ATP (fig. 4). This is possible only as long as Mg^{2+} is sufficient to form enough Mg^{2+}-ATP: APPNP which binds Mg^{2+} several times more strongly than ATP [13] cannot be replaced by free ATP.

iii) If the enzyme is saturated with Mg^{2+}-ATP at low Mg^{2+} levels, 0.3 mM APPNP acts as a chelator similar to EDTA and replaces ATP from the enzyme even at 2 mM ATP concentrations; the decay from ΔF_{max} to $2/3\ \Delta F_{max}$ occurs at a rate of k_3.

It is therefore concluded that the distribution of Mg^{2+} among competing substrates is determined by their binding constants and concentrations in the *solvent,* irrespective of whether the enzyme binds Mg^{2+} or not, and that the binding of competing substrates to the enzyme is again determined by their binding constants and concentrations in the solvent. There is no conversion from ATP to Mg^{2+}-ATP or vice versa at the enzyme. The dissociation constants increase in the order $K_{Mg\text{-}ATP} < K_{Mg\text{-}APPNP} < K_{ATP} < K_{APPNP}$.

4. Conclusions

The results can be summarized by the scheme

$$My_1 + Mg^{2+}\text{-}ATP \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} My_2 \cdot Mg^{2+} \cdot ATP \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} My_3 \times Mg^{2+} \cdot ADP \cdot P_i \rightleftharpoons \text{etc.}$$

in which My now stands for one of the two independent enzymatic centers of myosin which upon binding of Mg^{2+}-ATP as an entity is able to reach the long lived My_3-conformation presumably necessary for the physiological role of myosin. In the My_2 and My_3 conformations Mg^{2+} is buried in the interior of the enzyme–substrate complex.

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