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TRANSIENT KINETIC AND ISOTOPIC TRACER STUDIES OF THE MYOSIN ADENOSINE TRIPHOSPHATASE REACTION

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From transient kinetic studies of the Mg^{2+}-dependent adenosine triphosphatase of myosin subfragment 1, prepared from rabbit skeletal muscle, a seven-step mechanism has been proposed. Features of this mechanism include two-step processes for ATP and ADP binding in which the binary complex isomerizes in addition to a rapid nucleotide association step. In the case of ATP a large negative standard free energy change is associated with the isomerization. An overall rate-limiting isomerization of the myosin-product complex prior to product release has been identified. Studies on the mechanism of cleavage of ATP bound to the active site indicate the process is readily reversible and can account for the observation that more than one oxygen of the product phosphate arises from water. This proposal has been substantiated by the finding that the oxygen atoms of the γ-phosphoryl group of bound ATP also undergo extensive exchange with water.

INTRODUCTION

The identification of the chemical states of the actomyosin ATPase[1] during the force-generating cycle of muscle requires investigation of systems capable of generating tension. Chemical reactions in solution are, however, more amenable to study and serve as a basis for examining organized systems. Results from solution studies can provide important information and much attention has been paid to correlating these data with structural and mechanical studies (1). However, details of the elementary processes of the actomyosin ATPase have still to be evaluated. We have undertaken an investigation of the interaction between ATP and subfragment 1, the globular "head" region of the myosin molecule bearing one active site, with the aim of providing a framework for the study of more complex systems.

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[1] Abbreviations: ATPase, adenosine triphosphatase; Tris, tris (hydroxymethyl)aminomethane; M, myosin active site; ATP (γS), adenosine 5′-(3-thiotriphosphate); ATP (β, γ-NH), 5′-adenylyl-imidodiphosphate.

For the purpose of this paper the mechanism of this ATPase reaction is defined as the chemical identity of the intermediates of the pathway, together with the rate constants of their interconversion. The elucidation therefore demands direct observation of protein-ligand complexes during the course of the reaction and this, as with most enzyme reactions, requires the employment of rapid-reaction techniques (2). In principle, the transient processes of the reaction time course contain information pertaining to all the rate constants of the pathway. However, the number of rate constants which can be evaluated in practice, simply by mixing ligand with protein, is generally limited. Further resolution of the reaction mechanism may be achieved by the use of isotopically labeled substrates. Qualitatively, isotopes may be used to identify the position of bond cleavage in group transfer reactions, as exemplified by the finding that enzyme-catalyzed ATP hydrolysis generally results in water-oxygen incorporation into the product P_i and not ADP (3). Additional information is available from analysis of the rate of isotope incorporation in systems at equilibrium, in the steady state or during the transient state (4). This approach is particularly useful in the evaluation of the reverse rate constants of pathways, such as that of ATP hydrolysis, in which the overall equilibrium constant is large.

In this article we briefly discuss a kinetic model of the Mg^{2+}-dependent ATPase of subfragment 1 prepared from rabbit skeletal muscle based on rapid-reaction studies, then we review recent isotope incorporation studies pertinent to this mechanism.

A KINETIC MODEL

Our published work (5–9) concerning the elementary steps of the myosin ATPase which led to Equation 1 has been summarized (10) and can be consulted for experimental details.

$$\mathrm{M + ATP} \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} \mathrm{M.ATP} \overset{+\alpha H^+}{\underset{k_{-2}}{\overset{k_{+2}}{\rightleftharpoons}}} \mathrm{M^*ATP} \underset{k_{-3}}{\overset{k_{+3}}{\rightleftharpoons}} \mathrm{M^{**}ADP.P_i} \underset{k_{-4}}{\overset{k_{+4}}{\rightleftharpoons}} \overbrace{\mathrm{M^*ADP.P_i}}^{+1.0\,H^+} \underset{k_{-5}}{\overset{k_{+5}}{\rightleftharpoons}}$$

$$\mathrm{M^*ADP + P_i} \overset{-\alpha H^+}{\underset{k_{-6}}{\overset{k_{+6}}{\rightleftharpoons}}} \mathrm{M.ADP} \underset{k_{-7}}{\overset{k_{+7}}{\rightleftharpoons}} \mathrm{M + ADP}, \qquad [1]$$

where $K_1 = k_{+1}/k_{-1} = 4.5 \times 10^3\ M^{-1}$; $k_{+2} = 400\ s^{-1}$; $k_{-2} \leqslant 0.02\ s^{-1}$; $K_3 = 9$; $k_{+3} \geqslant 160\ s^{-1}$; $k_{+4} = 0.06\ s^{-1}$; $K_5 > 1.5 \times 10^{-3}$ M; $k_{+6} = 1.4\ s^{-1}$; $k_{-6} = 400\ s^{-1}$; $K_7 = 2.7 \times 10^{-4}$ M; and $\alpha = 0.23$ in a medium of 100 mM KCl, 5 mM $MgCl_2$, 50 mM Tris adjusted to pH 8.0 with HCl at 21°C. Isomers are distinguished by asterisks, the number of which relate approximately to the protein fluorescence enhancement compared with free myosin subfragment 1. Table I of the paper by Bagshaw and Trentham (8) lists the nomenclature used here and in previous publications.

The fundamental proposition of the Lymn-Taylor scheme (11, 12), that the cleavage reaction is rapid compared with the effective dissociation rate of the products, is necessary to account for the transient production of products when excess ATP is added to myosin. The identification of the major steady-state intermediate as $M^{**}ADP.P_i$ as distinct from the complexes formed by the addition of ADP and/or P_i to myosin resulted in

the extension of the Lymn-Taylor scheme by several investigators (5, 13–16). Trentham et al. (5) showed that at 21°C the steady-state complex contained both ADP and P_i since no transient formation of either free product was detected with a linked assay system. In any event, M*ADP is not the major steady-state intermediate under these conditions since it decays considerably faster than the steady-state ATPase rate and is spectrally distinguishable (relative fluorescence of the steady-state intermediate = 1.18 and M*ADP complex = 1.067 (8, 15)).

However, the mechanism of P_i dissociation is not fully characterized. P_i is a very poor inhibitor of the steady-state ATPase rate, which is consistent with the finding that the binding of P_i to M*ADP is described by an equilibrium dissociation constant of $>1.5 \times 10^{-3}$ M (8). A simplified scheme in which M**ADP.P_i decays by a compulsory ordered release of P_i at a rate of 0.06 s^{-1} (17) results in the association rate of P_i to form the species being uncharacteristically slow. A rate-controlling myosin-product isomerization step is therefore postulated prior to the rapid dissociation of P_i and ADP from M*ADP.P_i (5). However M*ADP.P_i remains poorly characterized since it is present at a very low concentration during the ATPase reaction, and attempts to generate it by addition of ADP and P_i to M are complicated by the formation of M*ADP + M.P_i (and probably M.ADP.P_i). The species M.P_i and M.ADP.P_i were characterized by the inhibition of the ATP and ADP association reactions in the presence of P_i (8).

While more general schemes of product dissociation can be written involving intermediates such as M.P_i, it was shown that the predominant pathway involves M*ADP, since, on reducing the temperature to 5°C, this species becomes a significant component of the steady-state complex (8). The extreme temperature sensitivity of the value of k_{+6} relative to k_{+4} was indicated by Martonosi and Malik (18) and at 5°C these rate constants are comparable in magnitude (k_{+6} = 0.07 s^{-1} and k_{+4} = 0.036 s^{-1}). At this temperature the fluorescence time course, on mixing excess ATP with M, shows a transient peak enhancement corresponding to the production of M**ADP.P_i at a concentration practically equal to the total protein concentration, $[M]_o$, followed by a decrease to an intermediate level corresponding to the steady-state concentrations of M**ADP.P_i and M*ADP. At 21°C no subsequent decrease in fluorescence enhancement was detected, showing that M**ADP.P_i is the predominant intermediate at this temperature (8).

The protein fluorescence enhancement observed on binding nucleotide to subfragment 1 has been instrumental in the elucidation of the kinetic mechanism of association. In addition, the use of ATP analogs, such as ATP (γS), which show no transient in bound-product formation (6) and therefore simulate the M*ATP state on binding to myosin, has been vital. When ATP, ADP, or ATP (γS) at a concentration of 20–100 μM is mixed with subfragment 1 ($\approx$ 4 μM) in a fluorescence stopped-flow apparatus, the fluorescence enhancement is exponential with a rate constant linearly dependent on nucleotide concentration. While the magnitude of the enhancements relative to free M are 1.18, 1.067, and 1.10 for each nucleotide, respectively, the apparent second-order rate constants for the processes are similar at $1 \times 10^6 - 2 \times 10^6$ $M^{-1} \cdot s^{-1}$. However, when the nucleotide concentration exceeds about 200 μM, the observed rate of the fluorescence change becomes independent of nucleotide concentration and reaches a limit of 300–400 s^{-1} at 21°C (9). Since, at least in the case of ADP and ATP (γS), the fluorescence change is a result of binding alone, the results necessitate a two-step association mechanism. While

alternative schemes are feasible (9), a simple pathway which satisfies the data involves isomerization of the binary complex:

$$M + X \underset{k'_{-1}}{\overset{k'_{+1}}{\rightleftharpoons}} M.X \underset{k'_{-2}}{\overset{k'_{+2}}{\rightleftharpoons}} M^*X \qquad \text{where X = nucleotide.} \qquad [2]$$

A hyperbolic relationship between the observed rate of the fluorescence change and [X] indicates that X and M.X remain in equilibrium throughout the production of the fluorescent M*X species (i.e., $k'_{-1} >> k'_{+2} + k'_{-2}$) and allows $k'_{+1}\,k'_{+2}/k'_{-1}$ to be equated with $1 \times 10^6 - 2 \times 10^6\ M^{-1}\ s^{-1}$ and k'_{+2} with 300–400 s^{-1}.

The binding of ATP is complicated by the subsequent cleavage reaction and the observed fluorescence change monitors the production of M*ATP + M**ADP.P_i. Within experimental sensitivity, the amplitude of the fluorescence change at [ATP] > 200 μM is the same as at lower ATP concentrations and the observed process is monophasic. It is possible there is an additional fluorescence change associated with the M*ATP ⇌ M**ADP.P_i transition within the signal to noise ratio of the stopped-flow spectrofluorimeter used. Recently J. F. Koretz and E. W. Taylor (personal communication) concluded that the observed rate of the fluorescence change and M**ADP.P_i production, as measured by the formation of P_i, were superposed over the [ATP] range studied at pH 8 and 20°C, so that k_{+3} may be very rapid and the rate of M**ADP.P_i production is limited by k_{+2}.

Additional evidence for the two-step mechanism of nucleotide association is provided by the time course of proton release. At pH 8.0 the hydrolysis of ATP results in the overall production of 1.0 H^+. A transient in H^+ production was observed on adding ATP to M, which was tentatively assigned to the formation of bound P_i since the rate of production showed a similar dependence on [ATP] as did the production of bound P_i (19). However, it was later shown that ADP and ATP (β,γ-NH), an ATP analog which is not cleaved by M, also release a transient H^+ on binding, but with these nucleotides there is no subsequent steady-state production of H^+ (8). Koretz and Taylor (personal communication) confirmed this observation and showed that the rate of transient H^+ release has the same dependence on [ADP] as [ATP] and superposes the fluorescence profiles. The magnitude of the transient proton release on adding ATP to M is about 0.23 mol H^+/mol subfragment 1 and is probably a result of pK changes of the amino acid residues during the M.ATP ⇌ M*ATP isomerization analogous to the Bohr effect in hemoglobin (20). During the overall ATPase reaction, a proton uptake of the same magnitude occurs at the M*ADP ⇌ M.ADP transition and therefore 1.0 mol H^+/mol subfragment 1 must be released at either step 4 or step 5. This was confirmed directly by following the time course of proton release during a single turnover of the ATPase in which ATP was added to a molar excess of M (8).

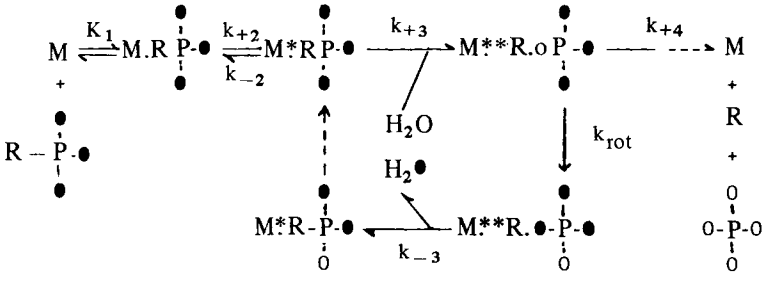
A single turnover experiment also proved useful in elucidating the mechanism of the cleavage reaction (7). When ATP is mixed with an excess of M, the fluorescence profile is biphasic, with a rapid increase in fluorescence corresponding to the binding reaction (controlled by $K_1 k_{+2}\,[M]_o$) and a subsequent decay during product release (controlled by k_{+4}). At the subfragment 1 concentration used, $k_{+3} >> K_1 k_{+2}\,[M]_o$, hence, once formed, M*ATP would be rapidly cleaved to M**ADP.P_i. When the binding phase is >99% complete (defined by time τ) it may be argued any remaining ATP is present as

M*ATP due to a dynamic equilibrium of the M*ATP ⇌ M**ADP.P_i transition. At this time, essentially all the nucleotide is distributed between M**ADP.P_i and M*ATP and hence the ratio of their concentration provides an estimate of K_3. By using [γ-^{32}P]-ATP it was shown 10% of the initial ATP remained at time τ, which decayed to 0% with the same rate as the release of products monitored by fluorescence. Hence K_3 is estimated as 9. Addition of a large excess of unlabeled ATP subsequent to τ had little effect on the decay of the labeled ATP, demonstrating essentially all the [γ-^{32}P]-ATP was tightly bound ($k_{-2} \leqslant 0.02\ s^{-1}$). If there was significant dissociation of the labeled M*ATP complex, the labeled ATP would become diluted by the unlabeled pool and would not be immediately turned over.

THE USE OF ISOTOPIC TRACERS

Levy and Koshland (21) demonstrated that when the myosin ATPase reaction was carried out in $H_2{}^{18}O$, the label was detected in the product P_i, in common with other ATPases (3). However, on quantitative analysis it was found that about three of the product P_i oxygens had incorporated ^{18}O. This phenomenon was termed "intermediate oxygen exchange" to distinguish it from the slow "medium oxygen exchange" observed between the oxygen of H_2O and added P_i (22). Further work on the intermediate oxygen exchange reaction by Sartorelli et al. (23) showed that, even during the first turnover of the myosin ATPase, the product P_i had undergone extensive exchange. When a large excess of ATP was added to myosin in $H_2{}^{18}O$, analysis of the remaining ATP after a substantial fraction had been hydrolyzed failed to detect ^{18}O incorporation. When the ATPase reaction was performed in $H_2{}^{16}O$ and the reaction was quenched with an acid solution containing $H_2{}^{18}O$ soon after the completion of the transient P_i production, no ^{18}O incorporation into P_i was observed (23). Since M**ADP.P_i would be a significant intermediate at the time of quenching, this result suggests that the complex comprises hydrolyzed products rather than a phosphorylated enzyme.

Among the possible explanations of these data, reversible metaphosphate formation by bound phosphate was suggested (23). However, in the light of the kinetic studies on the cleavage reaction indicating reversibility of the M*ATP ⇌ M**ADP.P_i transition and $^{32}P_i$ incorporation studies, it was proposed that the intermediate oxygen exchange reaction results from a reversible hydrolysis of bound ATP (7, 24). A simple scheme, derived from Equation 1, which explains the ^{18}O incorporation data is given in Equation 3,



[3]

where ○ and ● differentiate oxygen atoms arising from H_2O and ATP respectively, and R = ADP. The slow dissociation of ATP from M*ATP, controlled by k_{-2}, explains the negligible incorporation of ^{18}O in free ATP under the conditions used by Sartorelli et al (23).

In order to test the mechanism, experiments were undertaken in collaboration with P. D. Boyer and R. G. Wolcott to investigate the exchange of the oxygen atoms of the γ-phosphoryl group of protein -bound ATP during the ATPase reaction (25). ATP labeled with ^{18}O in the three terminal oxygens of the γ-phosphoryl group was mixed with an excess of subfragment 1 and the reaction was quenched when the binding phase was complete. The ATP and P_i arising essentially from M*ATP and M**ADP.P_i were isolated from the quenched mixture and their ^{18}O content analyzed. It was found that both species lost a significant amount of their label, compared with control samples in which [^{18}O]-ATP was added subsequent to the denaturation of the subfragment 1. The average loss for four experiments was 75% and in no case was less than 60%, and thus supports Equation 3. Kinetic analysis of Equation 3, using the estimated limit of k_{-3} of $> 18\ s^{-1}$ shows that the exchange reaction would recycle a sufficient number of times, before the products are released, so that all the oxygen atoms could exchange. The above data, together with results of analyzing P_i produced during the steady-state hydrolysis of ATP (21, 23), indicate that this is not so. Incomplete exchange could result from kinetic or structural restraints on the mechanism given in Equation 3. The ^{16}O incorporation rate might be limited by $H_2{}^{16}O$ accessibility. Alternatively, the P_i moiety may tumble slowly (i.e., $k_{rot} < k_{-3}$) or be restricted to rotation about one oxygen which is coordinated to the protein, thus preventing its exchange (7). Distinction of these effects may be achieved by following the time course of ^{18}O exchange in M**ADP.P_i during the transient phase.

Studies of $^{32}P_i$ incorporation into ATP tightly bound to myosin confirm that the hydrolysis reaction of ATP at the active site is readily reversible (26–28). These data also provide information concerning the equilibrium constants of the product binding and isomerization steps. However, discrepancies in the data do not allow the proposal of a unifying scheme. The results of Mannherz et al. (26) are kinetically competent with Equation 1 and allow the equilibrium constant K_2 to be evaluated knowing the overall equilibrium constant for ATP hydrolysis. It is important that their estimate is tested directly as has been attempted by Wolcott and Boyer (27, 28). In the presence of high ATP concentrations, they note $^{32}P_i$ incorporation into tightly bound ATP. In addition, they observe a slow continued incorporation which they interpret as a measure of the dissociation rate of bound ATP. This approach leads to a lower value for K_2. While the reasons for these differences constitute an important problem, these data substantiate that $K_2 > 10^4$, as calculated from the rate constants given in Equation 1.

The $^{32}P_i$ incorporation data, together with Equation 3, can account for the medium exchange phenomenon (22) between the oxygen atoms of free P_i and H_2O by the synthesis of M*ATP from products. In the presence of Mg^{2+} the exchange rate is slow and dependent on the concentration of myosin and P_i. With Mn^{2+} the exchange is faster and more readily detected (22, 29). The increased rate is possibly a reflection of the apparent higher affinity of P_i in this system (28, 29). As a consequence, a detailed study of the nucleotide dependence has been undertaken in the presence of Mn^{2+} (22, 29). The finding that the exchange is most prevalent with ADP and undetectable with ATP (β, γ-NH)

is consistent with the proposal that the exchange occurs at the active site by synthesis of ATP. ATP can substitute for ADP to some extent and this would be expected from the observation that in the presence of Mn^{2+}, a species analogous to M:*ADP is a significant component of the steady-state intermediate (29a).

DISCUSSION

The data from isotopic tracer studies are consistent with the proposal that the H_2O + M:*ATP ⇌ M:**ADP.P_i transition is readily reversible. Details of the mechanism of P_i binding and the value of the ATP dissociation constant are still unclear, although there is general agreement $k_{-2} < 0.02\ s^{-1}$.

The mechanism of Equation 1 fits well into the generalization about the kinetics of enzyme catalysis discussed by Gutfreund (2). The second-order steps of the first complex formation between enzyme and ligand are fast and nonspecific (and probably diffusion controlled where $k_{+1} \approx 10^8\ M^{-1}\ s^{-1}$). After substrate recognition an isomerization occurs to form a chemically reactive complex. Frequently these isomerizations are rate limiting, while the subsequent chemical processes are fast. An overall rate-limiting isomerization controlling product release has been noted in several cases (2).

Another feature of Equation 1 which may have general significance to enzyme catalysis is the change in reaction equilibria on the binding of the reactants to the active site. Contributory factors to the readily reversible hydrolysis of bound ATP probably include the reduced water activity and the retention of a proton in the M:**ADP.P_i complex at pH 8.0. The effective water concentration has direct bearing not only on the hydrolysis reaction, but also on the hydration shell of the reactants, which is proposed to make a significant contribution to the overall equilibrium of ATP hydrolysis (30). The consequence of proton retention by the ternary complex is also illustrated by the equilibrium constant of the lactate dehydrogenase reaction E.NAD^+ lactate ⇌ E. NADH.H^+ pyruvate, of 0.25 compared with the overall equilibrium constant for lactate-pyruvate conversion at pH 7.0 of 4×10^{-5} (31). Further examples of reversible hydrolysis reactions are quoted by Boyer et al. (32) and, in particular, common features of the myosin ATPase and the oxidative phosphorylation system are discussed.

Data presented in this paper show that there is a large negative standard free energy change associated with the formation of M:*ATP from M + ATP. While the corresponding steps of the actomyosin ATPase have not been elucidated in detail, the observation that actomyosin dissociates at $> 1000\ s^{-1}$ in the presence of ATP (12), yet the binding of actin to nucleotide free myosin is very tight (33), is consistent with the observed stability of M:*ATP. Solution studies therefore substantiate the proposal that a key role of ATP during the contraction cycle is in dissociating the crossbridge links from the actin filament (12, 34).

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