THE KINETIC MECHANISM OF THE MANGANOUS ION-DEPENDENT ADENOSINE TRIPHOSPHATASE OF MYOSIN SUBFRAGMENT 1

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1. Introduction

The paramagnetic properties of Mn(II) can be exploited to yield structural and dynamic information about the mechanism of enzyme action [1,2]. It is important, however, to determine the effect of substituting Mn(II) for the physiologically important divalent metal ion, in order to assess the pertinence of the magnetic resonance data. In this communication the kinetic pathway of the Mn(II) and Mg(II)-dependent ATPases of myosin subfragment 1 are compared.

Rapid reaction techniques have led to Equation (1) to describe the elementary steps of the ATPase in the presence of 5 mM Mg(II) [3,4].
(Me = divalent metal ion, S1 = myosin subfragment 1).

$$\mathrm{S1} + \mathrm{MeATP} \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} \mathrm{S1{\cdot}MeATP} \underset{k_{-2}}{\overset{k_{+2}}{\rightleftharpoons}}$$

$$\mathrm{S1{\cdot}{*}MeATP} \underset{k_{-3}}{\overset{k_{+3}}{\rightleftharpoons}} \mathrm{S1{\cdot}{*}{*}MeADP{\cdot}P_i} \underset{k_{-4}}{\overset{k_{+4}}{\rightleftharpoons}}.$$

$$\mathrm{S1{\cdot}{*}MeADP{\cdot}P_i} \underset{k_{-5}}{\overset{k_{+5}}{\rightleftharpoons}} \mathrm{S1{\cdot}{*}MeADP} + \mathrm{P_i} \underset{k_{-6}}{\overset{k_{+6}}{\rightleftharpoons}}$$

$$\mathrm{S1{\cdot}MeADP} \underset{k_{-7}}{\overset{k_{+7}}{\rightleftharpoons}} \mathrm{S1} + \mathrm{MeADP} \qquad (1)$$

where $K_1 = k_{+1}/k_{-1} = 4.5 \times 10^3\ \mathrm{M^{-1}}$, $k_{+2} = 400\ \mathrm{s^{-1}}$, $k_{-2} \leq 0.02\ \mathrm{s^{-1}}$. $k_{+3} \geq 160\ \mathrm{s^{-1}}$, $K_3 = 9$, $k_{+4} = 0.06\ \mathrm{s^{-1}}$, $K_5 > 10^{-3}$ M, $k_{+6} = 1.4\ \mathrm{s^{-1}}$, $k_{-6} = 400\ \mathrm{s^{-1}}$ and $K_7 = 2.7 \times 10^{-4}$ M in a medium of 0.1 M KCl, 50 mM Tris adjusted to pH 8.0 with HCl at 21°C. Isomers are distinguished with asterisks, the number of which relates approximately to the protein fluorescence enhancement compared with free subfragment 1. Under these conditions, the steady-state rate of ATP hydrolysis is controlled essentially by k_{+4} and the steady-state complex comprises an equilibrium mixture of S1·*MgATP and S1·**Mg-ADP·P_i. However, at 5°C the rate constant, k_{+6}, is markedly reduced so that S1·*MgADP also contributes to the steady-state complex [3]. The mechanism was established from the time course of the protein fluorescence change on addition of ATP and ADP. Similar procedures are used to identify intermediates of the Mn(II)-dependent subfragment 1 ATPase.

2. Methods

Subfragment 1, a protein containing one ATPase site derived from the controlled proteolysis of myosin, and nucleotides were obtained as described previously [3,5]. Rapid fluorescence changes were observed using a stopped-flow apparatus [6]. Other fluorescence measurements were obtained with a Perken-Elmer MPF-2A spectrofluorimeter equipped with a temperature controlled cell holder. The interpretation and extraction of rate constants from the data have been discussed in detail elsewhere [3,4]. A brief summary is provided here. When an excess of ATP is added to subfragment 1 a rapid protein fluorescence enhancement, F_1†, is observed correspond-

† Fluorescence values for F_1, F_2 are F_3 are expressed relative to free subfragment 1 fluorescence.

ing to S1·* *MeADP·P_i formation. The rate of this process is described by $K_1\ k_{+2}$ [ATP] at low ATP, but at ATP $>>$ K_1^{-1} the rate is independent of ATP and is essentially described by k_{+2}. An immediate exponential decay of the fluorescence, F_1, follows to the steady-state fluorescence level, F_2, with a rate constant $k'_{+4} + k_{+6}$ (where $k'_{+4} = k_{+4}/(1 + K_3^{-1})$), although the amplitude of this process $\rightarrow 0$ when $k'_{+4} << k_{+6}$. The fluorescence remains at F_2 while the ATP is hydrolyzed with a steady-state rate of $k'_{+4}\ k_{+6}/(k'_{+4} + k_{+6})$. Finally the fluorescence decays due to ATP exhaustion and ADP inhibition to F_3, which corresponds to the fluorescence of S1·*MeADP. F_2 reflects the composition of the steady-state complex and equals $(k_{+6}F_1 + k'_{+4}F_3)/(k'_{+4} + k_{+6})$. When subfragment 1 is added in excess of ATP a single turnover occurs in which an initial rapid fluorescence enhancement is observed corresponding to S1·**MeADP·P_i formation, followed by a decay in fluorescence controlled essentially by k'_{+4}. When an excess of ADP is added to subfragment 1 a fluorescence enhancement is observed, F_3, with a rate constant k_{-6} [ADP]/K_7 at low ADP and k_{-6} at high ADP (i.e. ADP $>>$ K_7). Approximations have been made to obtain these simple relationships from eqn (1) and the original publications should be consulted as to their nature and validity [3,4].

3. Results

The steady-state rate of ATP hydrolysis by myosin is an order of magnitude faster in the presence of Mn(II) compared with Mg(II) [7]. Hence if eqn (1) applies to the Mn(II)-dependent ATPase k_{+4} must be accelerated. When an excess of [γ-^{32}P] ATP is added to myosin subfragments and the reaction is quenched with acid a transient in P_i production is noted with both metal ions, showing the rate determining step occurs after the cleavage reaction [8,9].

Fluorescence studies were performed to establish the mechanism in more detail. On mixing an excess of ATP with subfragment 1 in a stopped-flow device under the conditions described for eqn (1), but in the presence of 5 mM Mn(II) a similar enhancement of fluorescence was observed as with Mg(II). The apparent second order rate constant [4] for the

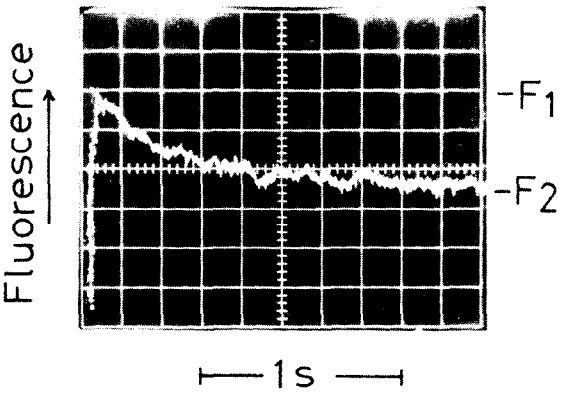

Fig.1. Stopped-flow record of the protein fluorescence on mixing an excess of ATP with subfragment 1 in the presence of Mn(II) at 21°C. One syringe contained 200 μM ATP and the other 5.6 μM subfragment 1 (reaction chamber concentrations). Both syringes contained 0.1 M KCl, 5 mM $MnCl_2$ and 50 mM Tris adjusted to pH 8.0 with HCl. The fluorescence remained at F_2 during the steady-state hydrolysis of ATP as shown by the trace in fig.2 obtained at 21°C. The rate constant for the exponential decrease in fluorescence from F_1 to F_2 was 2.5 s^{-1}.

process, $K_1\ k_{+2}$ was 2.3×10^6 $M^{-1} \cdot s^{-1}$. However, in contrast to the Mg(II)-dependent ATPase at 21°C the peak transient fluorescence F_1 was observed to decay immediately (fig.1). The rate constant of this exponential process was 2.5 s^{-1} and may be equated with $k_{+4} + k_{+6}$. When a single turnover of ATP was examined by mixing ATP with an excess of subfragment 1 [5], the rate of the initial phase of the fluorescence enhancement corresponding to ATP binding and cleavage was slightly faster in the presence of Mn(II) as expected from the apparent second order binding constant. However, the second phase corresponding to the decay of the product complex, S1·* *MeADP·P_i to S1·*MeADP was 1 s^{-1} in the presence of Mn(II) i.e. k'_{+4} is 20 times that with Mg(II) [3]. The value of k_{+6} of 1.5 s^{-1} calculated from these data suggests substituting Mn(II) for Mg(II) has little effect on the process controlled by k_{+6}. This rate constant shows an extreme temperature dependence in the presence of Mg(II). It is therefore of interest to study the effect of temperature on the Mn(II)-dependent ATPase. Fig.2 shows a comparison of the time course of fluorescence when an excess of ATP is added to subfragment 1 in the presence of 1

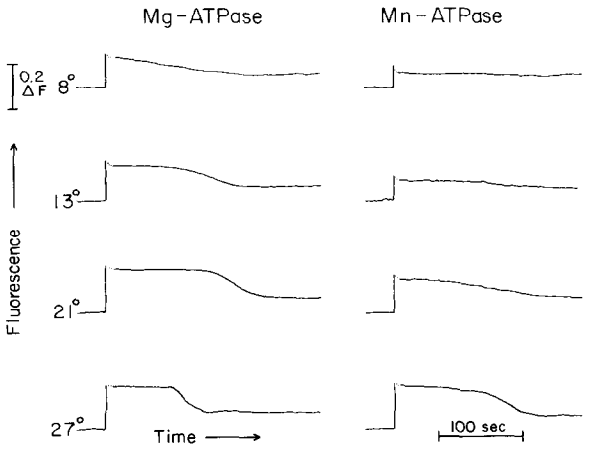

Fig.2. The fluorescence time course during the hydrolysis of an excess of ATP by subfragment 1 in the presence of Mg(II) or Mn(II) at various temperatures. Excitation light was at 295 nm and the emission was monitored at 340 nm. The initial fluorescence reading is that of 1.6 μM subfragment 1 in 0.1 M KCl, 50 mM Tris adjusted to pH 8.0 (at 22°C) with HCl, and either 1 mM $MgCl_2$ or $MnCl_2$. Some drift in the reading was noted, particularly in the presence of Mn(II) when the solution was exposed to u.v. light for longer than 300 s. For this reason ATP concentrations (see table 1) were selected to give approximately the same steady-state life-time in all assays. The fluorescence could be recorded 10 s after ATP addition and therefore the transient fluorescence F_1 noted in figure 1 was not observed in these records. The fluorescence when all the ATP had been hydrolyzed, F_3 was 1.06 ± 0.01 under all conditions. These data are analyzed in table 1.

mM Mg(II) or Mn(II). While the transient phase fluorescence, F_1, could not be resolved when ATP was added manually, the reading of the fluorimeter was more stable over the time period required to hydrolyze the ATP. The fluorescence when all the ATP had been hydrolyzed was 1.6 ± 0.01 under all conditions and corresponds to the S1·*MeADP enhancement, F_3. It is apparent from table 1, the transition from the condition where the steady-state fluorescence reflects that of S1·**MeADP·P_i (i.e. $F_2 = F_1 = 1.19$) to that of S1·*MeADP (i.e. $F_2 = F_3 = 1.06$) occurs over a range of about 20°C in both the Mg(II) and Mn(II) dependent ATPase. However, the mid-point of the transition, when $k'_{+4} = k_{+6}$ occurs at about 15°C in the presence of Mn(II) but at about 5°C in the presence of Mg(II) [3]. At 21°C k'_{+4} and k_{+6} are 0.7 s^{-1} and 1.6 s^{-1} respectively for the Mn(II) system as calculated from F_2 and the steady-state rate given in table 1. These values are in reasonable agreement with the stopped-flow data above, obtained with a different subfragment 1 preparation. The data suggest the value of k_{+6} shows a similar marked temperature dependence in the presence of Mg(II) or Mn(II). (Activation energy = 130 kJ/mol, not 300 kJ/mol as erroneously reported [3]). The similarity of the S1·*MgADP and S1·*MnADP complexes is also reflected in their apparent second order rate constants of formation k_{-6}/K_7 of 2×10^6 and 1.5×10^6 $M^{-1} \cdot s^{-1}$ respectively as measured by the fluorescence stopped-flow technique [4]. Hence the apparent dissociation equilibrium constant of the S1·*MeADP complex, K_6K_7, is about 1×10^{-6} M for both metal ions at 21°C.

Table 1
Effect of temperature on the steady-state fluorescence of the Mg(II) and Mn(II) subfragment 1 ATPase

Temp °C	Mg(II) – ATPase F_2	k'_{+4}/k_{+6}	$[ATP]_0$ μM	k_{cat} s^{-1}	Mn(II) – ATPase F_2	k'_{+4}/k_{+6}	$[ATP]_0$ μM	k_{cat} s^{-1}
8	1.15	0.45	3.3	—	1.07	12	33	—
13	1.17	0.18	6.6	0.019	1.11	1.4	66	—
21	1.18	≈0.08	17	0.041	1.15	0.45	167	0.48
27	1.19	→ 0	17	0.066	1.19	→ 0	333	1.06

F_2 is the fluorescence enhancement observed during the steady-state hydrolysis of ATP. k'_{+4}/k_{+6} was calculated from F_1, F_2 and F_3, assuming $F_1 = 1.19$ under all conditions (see Methods). $[ATP]_0$ was the concentration of ATP added to initiate the reactions shown in fig.2. From the $[ATP]_0$, the subfragment 1 concentration and the life-time of the steady-state fluorescence, the steady-state rate, k_{cat}, can be calculated. This method is not applicable when $F_2 \rightarrow F_3$ or when ADP inhibition is extensive as indicated by a reduction in the value of F_2 before the ATP approaches exhaustion [3]. For the Mg(II)-ATPase $F_2 = 1.12$ at 5°C. i.e. $k'_{+4}/k_{+6} \approx 1$ [3].

4. Discussion

The mechanisms of the Mn(II) and Mg(II)-dependent ATPases are basically similar and can be rationalized in terms of eqn (1). There is a notable reduction in the life-time of the S1·**MnADP·P_i complex compared with S1·**MgADP·P_i as indicated by the value of k_{+4} and accounts for the increased steady-state rate and reduced intermediate oxygen exchange with water [10]. Medium oxygen exchange studies show differences in the presence of Mn(II) and Mg(II) which may reflect changes in the values of K_5 and k_{-4} as well as the change in k_{+4} [10,11].

A consequence of the larger value for k_{+4}, but similar value for k_{+6} is that S1·*MeADP comprises a greater proportion of the steady-state complex in the Mn(II)-dependent ATPase. This conclusion was also reached by Yazawa et al. [8] and Hozumi and Tawada [9] using u.v. difference spectroscopy to identify the intermediates. However, these authors favour two pathways of ATP hydrolysis to explain their results. Their principle evidence for extending the mechanism arises from the stoichiometry of the transient P_i production of less than 1 mole/active site [8,9]. However, besides errors in estimating the active site concentration, this may also arise due to an equilibration between the S1·*MeATP and S1·**MeADP·P_i complexes in a single pathway [5]. Such an equilibrium probably exists in the Mn(II)-dependent ATPase since the product P_i contains more than one oxygen from the water [10] as a consequence of the reversibility of step 3 [12]. In the single turnover experiment reported here it was shown S1·**MnADP·P_i decays sufficiently fast to account for the steady-state rate of ATP hydrolysis. The decay in fluorescence after several turnovers of ATP is a complex function due to inhibition by ADP [3], and its apparent rate constant cannot be related simply to S1·**MeADP·P_i breakdown [9]. The profile of the fluorescence change during the approach to the steady-state is adequately explained by a linear mechanism and supports the proposal that S1·*MeADP is an intermediate within the catalytic cycle [3].

The existence of S1·*MnADP as the steady-state complex in the Mn(II)-dependent ATPase has also been demonstrated by the inhibition of the transient P_i production on pre-addition of ADP to the myosin [8,9]. Using this technique Hozumi and Tawada [9] claim there is a sharp transition in the composition of the steady-state complex at 10°C. The steady-state fluorescence shows k_{+6} is markedly temperature dependent but no such transition is indicated, nor does the transition appear particularly sharp when the steady-state rate is measured [9].

While a study of the effect of the divalent metal ion yields information pertinent to the mechanism of catalysis the main objective of this investigation was to establish whether Mn(II) is a good analogue of Mg(II). These results show the nucleotide binding reactions are very similar, as judged by the values of K_1 k_{+2}, k_{+6} and k_{-6}/K_7. The structural and dynamic information about the S1·*MnADP complex obtained by magnetic resonance techniques is therefore applicable to the physiologically important S1·*MgADP complex [13]. Mn(II) is known to mimic Mg(II) in accelerating the shortening of actomyosin threads in the presence of ATP and further substantiates the close analogy between these metal ions [14].

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