ON THE STRUCTURE OF THE MYOSIN–ADP–Mg COMPLEX

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1. Introduction

Two methods have emerged as useful tools in determining the structures of enzyme–metal–nucleotide complexes:

(i) The use of exchange-inert metal complexes of nucleotides as analogs of the rapidly interconverting Mg^{2+} complexes [1–3];

(ii) The change of enzyme specificity for the diastereomers of nucleotide phosphorothioates on changing the nature of the divalent metal ion [4,5].

Where comparisons have been made, the two approaches have led to similar conclusions. However, both methods must be used with caution, since there are several possible sources of error. The case of myosin is proving particularly difficult, despite the apparently clear-cut results in [6] and this is discussed below.

2. Materials and methods

Myosin subfragment 1 (S1) was prepared as in [7] by proteolytic cleavage of insoluble rabbit myosin. For most experiments reported here, S1–A1 was used. Thiophosphate analogs were prepared as in [8,9]. Chromium derivates of ADP were prepared according to [10] and characterized by their inhibition of hexokinase. β-Monodentate CrADP exhibited a K_i-value of 4 μM at pH 7.0, and α,β-bidentate CrADP inhibited much more weakly. ε-ADP was prepared by S1-catalyzed hydrolysis of ε-ATP, which was prepared as in [11].

Stopped-flow measurements of changes in the intrinsic fluorescence of S1 were made using a Durrum D 117 rapid mixing system [9]. Static fluorescence titrations were performed using a Perkin-Elmer MPF 3 spectrophotometer. The binding of ε-ADP caused a 20% decrease in the tryptophan fluorescence of S1 (λ_{ex} = 297 nm, λ_{em} = 340 nm), and this was used as a signal for titrations in the presence and absence of other nucleotides. Vanadate experiments were performed as follows: S1, ADP, and divalent metal ions were mixed at concentrations sufficiently high to ensure >95% saturation of S1 with metal–ADP complex. Sodium vanadate (1 mM) was added, and the Mg^{2+}–ATPase activities of aliquots of the resulting solution were assayed at recorded time intervals. These were compared with those of a control incubation in the absence of vanadate.

The rate and extent of decomposition of β-monodentate CrADP was measured using two coupled enzyme assays. ADP which was formed was either phosphorylated by pyruvate kinase in a system often used for ATPase assays, or was hydrolyzed by alkaline phosphatase to AMP and adenosine which were deaminated to IMP and inosine accompanied by a change in the molar extinction coefficient of −8200 cm^{-1} at 265 nm.

Unless otherwise stated, all experiments were carried out at 22–24°C in Tris–HCl (50 mM) pH 8.0 and KCl (50 mM). For experiments in the presence of divalent metal ions, $MgCl_2$ (5 mM), $CoCl_2$ (1 mM) or $Co(NO_3)_2$ (1 mM) were included.

3. Results

The association constants of ADP(α-*S*)A and ADP(α-*S*)B to myosin are similar (10^5 M^{-1} cf. 4×10^4

Abbreviations: ADP(α-*S*)A and ADP(α-*S*)B, A and B diastereomers [8] of adenosine 5¹-*O*-(1-thiodiphosphate); ATP(α-*S*)A and ATP(α-*S*)B, A and B diastereomers of adenosine 5¹-*O*-(1-thiotriphosphate); ATP(β-*S*)A and ATP(β-*S*)B, A and B diastereomers of adenosine 5¹-*O*-(2-thiotriphosphate); β-CrADP, β-chromium monodentate complex of ADP; α,β-CrADP, α,β-chromium monodentate complex of ADP; ε-ADP, 1,6-*N*-ethenoadenosine 5¹-*O*-triphosphate

M^{-1}) as determined using transient-kinetic methods [9]. A steady state method [6] also showed a difference of a factor of ~2. Here, the association constants were redetermined using a method based on the inhibition of ϵ-ADP binding to S1 as determined from the degree of quenching of the intrinsic protein fluorescence. Under the conditions used, ϵ-ADP exhibited a K_i value of 2 μM, and both ADP(α-S)A and ADP-(α-S)B had values of 20 μM. Thus, the diasteromers could not be distinguished in terms of their affinity for S1 in the presence of Mg^{2+}. For this reason, a reversal (or lack) of preference for the diasteromers in the presence of Co^{2+} or Cd^{2+} could not be used to deduce the structure of bound metal complex. A slight preference of the A over the B isomer with both Mg^{2+} and Co^{2+} was reported [6], but this cannot be taken as definitive evidence because of the small size of the effect.

In [6], β-CrADP interacted strongly with S1 whereas α,β-CrADP did not. We were not able to confirm this result [12]. We attempted to measure the dissociation kinetics of β-CrADP from its complex with S1. After mixing S1 with 20 μM β-CrADP at pH 8.0 in the absence of Mg^{2+}, and subsequent displacement with MgATP in the stopped-flow spectrophotometer, it was found that the rate of binding of ATP was not different from that observed in an experiment where β-CrADP was omitted from the enzyme solution. If Mg^{2+} (5 mM) and β-CrADP were included with the S1, a much slower binding of MgATP was observed, at a rate which corresponded to the rate of dissociation of MgADP from its complex with S1 (1.9 s^{-1} at 23°C). At pH 7.0, similar effects were observed, except that the amplitude of the slow phase in the experiment in which Mg^{2+} was included was smaller, and variable. These results suggested that ADP impurities in the β-CrADP were binding to S1 in the presence of Mg^{2+}.

The question of the strength of binding of β-CrADP and α,β-CrADP was also investigated using the competition titration technique. At pH 7.0, the binding of ϵ-ADP to S1 was not affected by β-CrADP concentrations of up to 100 μM, except in cases where the β-CrADP was preincubated at pH 7.0 for $\geqslant$ 10 min (the pH of β-CrADP solutions after column purification is ~2.5–3.5) (fig.1). This effect was more pronounced at pH 8.0, and a clear time dependence of the inhibitory power could be observed. The increase of inhibitory power on preincubation was not dependent on the presence of S1 during the preincubation period,

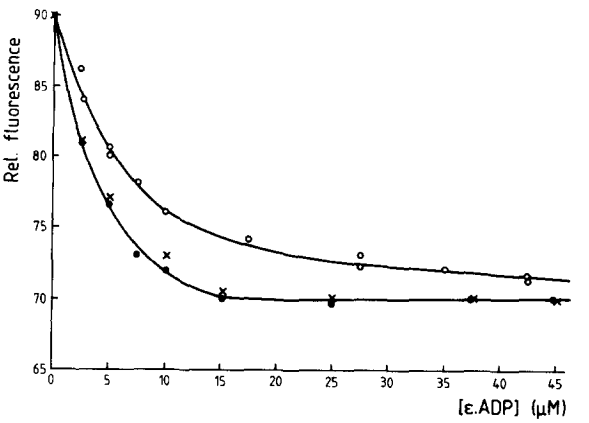

Fig.1. The effect of preincubation of –CrADP at pH 7.0 on the inhibition of ADP binding to S1: (•) titration in the absence of β-CrADP; (×) immediately after adding 75 μM β-CrADP; (○) after 20 min preincubation of 75 μM β-CrADP in the titration buffer (50 mM imidazole–HCl, 50 mM KCl, 5 mM $MgCl_2$, pH 7.0, 22°C).

showing that the time lag was not caused by slow formation of a stable complex between the enzyme and nucleotide. Since these results suggested that instability of β-CrADP was a serious problem, possible ADP production on incubating solutions of the analog at pH 7.0 and pH 8.0 was investigated. Using two different ADP-assay systems, it was found that decomposition of β-CrADP to ADP occurred to an extent (30% at pH 7.0, 58% at pH 8.0) and rate ($t_{1/2}$ = 50 min at pH 7.0, 8 min at pH 8.0 and 24°C) which can account for the inhibitory power exhibited by solutions of β-CrADP at neutral or alkaline pH. It is therefore concluded that neither β-CrADP nor α,β-CrADP, which also failed to inhibit ϵ-ADP binding to S1 confirming [6], are good analogs of the MgADP complex bound in the myosin–ADP–Mg state of the myosin ATPase. These analogs can therefore not give information on the coordination of Mg^{2+} to the phosphate groups of ADP in complexes with S1.

Because of the lack of suitability of the Cr–ADP complexes for the determination of the structure of MgADP when the latter is bound to S1, studies using the thiophosphate analogs of ADP were extended. Although ADP(α-*S*) diasteromers A and B have similar affinities for S1 in the presence of Mg^{2+}, they can be easily distinguished by the kinetics of the interaction [9]. The rate of release of MgADP(α-*S*)A from its complex with S1 is relatively rapid (~13 s^{-1} cf. 2 s^{-1} for MgADP), and its association rate constant (~10^6

Table 1
Rates of dissociation of metal complexes of ADP(α-*S*) diastereomers from S1

Nucleotide	Metal ion	k_{diss} (s^{-1})
ADP(α-*S*)A	Mg^{2+}	13
ADP(α-*S*)B	Mg^{2+}	2.3×10^{-2}
ADP(α-*S*)A	Co^{2+}	>0.5
ADP(α-*S*)B	Co^{2+}	2.7×10^{-2}
ADP(α-*S*)A	Cd^{2+}	>0.5
ADP(α-*S*)B	Cd^{2+}	3.2×10^{-2}
ADP	Mg^{2+}	1.9
ADP	Cd^{2+}	>0.5

$M^{-1} . s^{-1}$) is similar to that for MgADP. In contrast, MgADP(α-*S*)B is released extremely slowly (k_{diss} = 2.3×10^{-2} s^{-1}) and is bound correspondingly slowly. Thus, ADP(α-*S*)A shows the more nearly ADP-like binding to S1, and ADP(α-*S*)B binds to S1 in an anomalous fashion, which cannot be interpreted in an obvious manner. However, if the required complex of MgADP for binding to S1 is the α,β-bidentate, then it would be expected that substitution of Mg^{2+} by Co^{2+} or Cd^{2+} should lead to a reversal of this behaviour.

The results of experiments on the rate of release of diastereomers of ADP(α-*S*) in the presence of various metal ions are shown in table 1. It can be seen that, irrespective of the nature of the metal ion, the B diastereomer is released much more slowly than the A isomer. This indicates that the nature of the metal coordination of the diastereomers in their complexes with S1 are similar. This was confirmed by the formation of a stable complex between S1, vanadate and MgADP [13], MgADP(α-*S*)A, CoADP or CoADP(α-*S*)A but not between S1, vanadate, and MgADP(α-*S*)B or CoADP(β-*S*)B (table 2). Although it was not possible to experiment on vanadate inhibition in the presence of Cd^{2+} complexes of nucleotides (due to inhibition by Cd^{2+}), the rates of release of CdADP(α-*S*)A and CdADP(α-*S*)B from their complexes with S1 could be determined. As shown in table 1, the B isomer is released anomalously slowly, as for Mg^{2+} and Co^{2+}. Since Cd^{2+} exhibits a marked preference for sulphur over oxygen as ligands, this is a strong indication that the metal ion is not coordinated to the α-phosphate in the S1–metal–ADP complex.

Table 2
Formation of a stable complex between S1, diphosphate, metal ion and vanadate [17]

Nucleotide	Metal ion	Inhibition by vanadate
ADP	Mg^{2+}	+
ADP	Co^{2+}	+
ADP(α-*S*)A	Mg^{2+}	+
ADP(α-*S*)B	Mg^{2+}	–
ADP(α-*S*)A	Co^{2+}	+
ADP(α-*S*)B	Co^{2+}	–

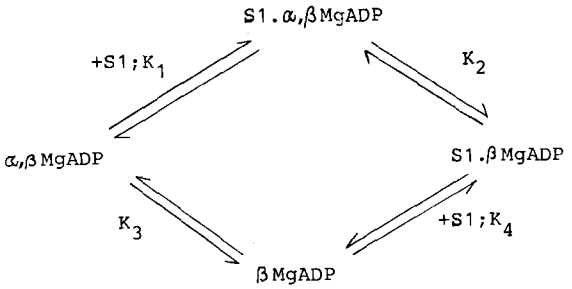

Fig.2. Two possible mechanisms of binding of MgADP to S1.

In fig.2, two pathways are shown for the binding of MgADP to myosin, assuming that the metal–nucleotide complex exists predominately in the α,β-bidentate form in solution. The results discussed above cannot distinguish between these possibilities.

In fig.3 an attempt is made to incorporate the find-

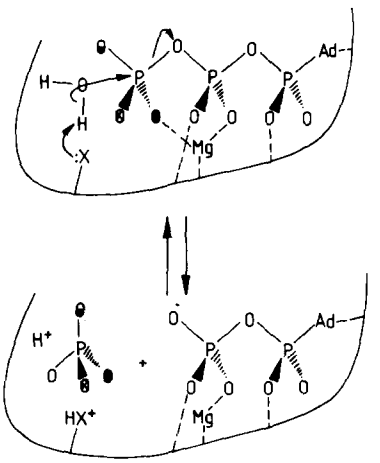

Fig.3. The binding of ATP and ADP to the active site of myosin and the cleavage of the terminal phosphate. The postulated modes of binding and hydrolysis are based on results reported here elsewhere, as discussed in the text. The continuous black line represents the active site, and X is a basic side group.

ings into schematic models for the binding of MgADP and MgATP to the active site of myosin. The evidence presented above for the lack of coordination of the α-phosphate group oxygen to Mg^{2+} in myosin–ADP–Mg makes our previous suggestion of the presence of such an interaction in myosin–ATP–Mg unlikely to be correct [9]. We thus assume that MgATP binds as the β,γ-bidentate complex (configuration determined from the known absolute stereochemistry of the preferred diastereomer of ATP(β-*S*), namely ATP(β-*S*)A, see [9]. This interpretation is strengthened by results on the effect of the nature of divalent metal ions used in steady-state experiments on the S1-catalyzed hydrolysis of the diastereomers of ATP(β-*S*). These experiments indicate that Δ β,γ-MgATP is the substrate [17]. The interaction of the enzyme with the α-phosphate group is included, since this must be responsible for the preferred binding of the A isomers of ADP(α-*S*) and ATP(α-*S*) [9]. The interaction of the β-phosphate group oxygen of ATP with the enzyme is assumed because of the very poor interaction even of the preferred diastereomer of ATP(β-*S*) with S1 [9]. The interaction between the metal ion and the enzyme is included because of the lack of interaction of β-monodentate CrADP with S1. As suggested [14], the inability of chromium to exchange its water ligands with enzyme ligands may be responsible for the weak interaction of such analogs with S1. The diagram also shows the P_i produced on cleavage of the terminal phosphate group and indicates the fate of the 3 oxygen atoms attached to this group as shown by experiments which indicate an SN_2-type attack of water in the myosin ATPase [15], presumably occurring via a pentacovalent phosphorous intermediate. Specific strong interactions of enzyme or metal ion with individual oxygens of the P_i in this state (i.e., the first state on the reaction pathway in which the covalent bond between the β- and γ-phosphates is cleaved) is unlikely based on the evidence that all 4 oxygen atoms are equivalent on the time-scale in which hydrolysis occurs, as shown by ^{18}O-exchange experiments [16].

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