STRUCTURE OF THE METAL–NUCLEOTIDE CHELATE IN THE MYOSIN–PRODUCT COMPLEX

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1. Introduction

Under physiological conditions, both ATP and ADP exist as a rapidly equilibrating mixture of Mg^{2+}–nucleotide chelates [1], but ATP- and/or ADP-utilizing enzymes are presumably specific for a particular metal–nucleotide coordination isomer [2–4]. In order to determine the metal–nucleotide chelate for which various enzymes are specific, two fruitful approaches have been developed. The first method, pioneered by Cleland and coworkers [1,3], involves the use of exchange-inert metal–nucleotide complexes; e.g., the interaction of yeast hexokinase with the coordination isomers of $Co(NH_3)_4ATP$ and CrADP, respectively, showed that the substrate of this enzyme is the β,γ-bidentate metal–ATP chelate [5] and that the product is the β-monodentate metal–ADP chelate [3]. (See also [6–8] for other examples.) The second method for determining whether the metal ion is liganded to a given phosphate moiety involves the use of nucleoside phosphorothioates [4,9]. Thus, Jaffe and Cohn [10,11] have investigated the substrate specificity of yeast hexokinase for the diastereomers of ATPαS and ATPβS as a function of metal cation and have concluded that the metal is liganded to the β-phosphorus but not to the α-phosphorus of enzyme-bound ATP. (See [12–14] for other examples.)

Myosin, the major protein constituent of muscle, catalyzes the hydrolysis of ATP to ADP [15–17]. The presence of a divalent metal ion such as Ca^{2+}, Mn^{2+}, Co^{2+}, or Mg^{2+} [18] or a monovalent ion such as NH_4^+, K^+ or Rb^+ [19] is necessary for activity. Bagshaw and Trentham [20] have shown that the metal ion is associated with ATP in the myosin–substrate complex and that it is released as a complex with ADP. In addition, it has been shown that the metal, when complexed to ADP at the active site, is highly sequestered from bulk solvent [21,22]. Ramirez et al. [23] have postulated that myosin is specific for the Λ α,β-bidentate–metal chelate of ATP whereas Goody and Hofmann [24] have concluded that the substrate is the Δ *exo* tridentate isomer. Preliminary evidence from this laboratory [25] indicates that the metal is not liganded to the β-phosphorus of the substrate. Here we investigate the inhibition of myosin ATPase by phosphorothioate analogs and Cr^{3+} complexes of ADP in order to determine the structure of the metal–ADP product. Results indicate that ADP is formed as the β-monodentate metal chelate.

Abbreviations: ATPαS and ATPβS, adenosine 5′-*O*-(1-thiotriphosphate) and 5′-*O*-(2-thiotriphosphate), respectively; ADPαS, A and B, the two diastereomers of adenosine 5′-*O*-(1-thiodiphosphate); ADPβS, 5′-*O*-(2-thiodiphosphate); ATPase, adenosine triphosphatase

2. Materials and methods

ATP and ADP were purchased from Pharma-Waldorf, [γ-^{32}P]ATP was obtained from Amersham Buchler, and ADPβS was supplied by Boehringer Mannheim. Rabbit skeletal myosin was isolated and quantitated as in [26].

The two diastereomers of ADPαS were prepared from the corresponding ATPαS isomers [27] by digestion with myosin at room temperature in a solution containing ~5 mM ATPαS, 25 mM Tris–HCl, (pH 8.0), 0.1 M KCl, 10 mM $CaCl_2$, and 1 mg enzyme/ml. The reaction mixture was chromatographed at 4°C

through a DEAE–Sephadex A-25 column using a linear gradient of 0.1–1.0 M triethylammonium bicarbonate (pH 7).

The β-monodentate chelate of CrADP was prepared by the procedure in [3,28] by heating a solution 20 mM each in $CrCl_3$ and Na_2HADP for 1 min at 80°C. The αβ-bidentate isomers of CrADP were prepared by the method used to make the monodentate isomer, except that the heating time was increased from 1–10 min [28]. The CrADP isomers were characterized by their inhibition or lack thereof against yeast hexokinase [3,28].

All myosin assay solutions contained in 2 ml: 1 μg enzyme/ml; 25 mM imidazolium–HCl, (pH 7.0 or 8.0); 0.5 M NaCl; and either 5 mM $MgCl_2$ or 1 mM $CoCl_2$. Enzymatic hydrolysis of ATP was measured as in [26,29].

3. Results and discussion

As noted in [29], the steady-state kinetics of myosin is biphasic, showing substrate activation at high substrate concentrations. In order to investigate the inhibition of myosin ATPase at the active site by ADP and its analogs, it was necessary to work at <0.5 μM substrate and <100 μM inhibitor. (At high substrate and inhibitor concentrations, ADP inhibits myosin K^+-ATPase in a predominantly noncompetitive manner [data not shown] with K_i = 20 mM). Fig.1 shows some representative data for the inhibition of myosin Mg^{2+}-ATPase by various ADP phosphorothioate analogs. Similar data were obtained for myosin's Co^{2+}-ATPase activity. Table 1 summarizes the kinetic parameters which have been obtained. (Myosin showed no ATPase activity in the presence of Ni^{2+}, Zn^{2+}, or Cd^{2+}.)

Both diastereomers of ADPαS are good inhibitors of myosin ATPase, with the K_i value for the B isomer being about twice that for the A isomer regardless of whether the metal ion is Mg^{2+} or Co^{2+}; i.e., myosin's inhibitor selectivity with respect to the two diastereomers of ADPαS appears to be independent of the metal ion. This may be taken as prima facie evidence that the metal does not coordinate to the α-phosphorus in the enzyme–ADP complex [4,9]. In order to provide conclusive evidence for this, lack of reversal with a metal ion such as Cd^{2+}, which strongly prefers chelation to sulfur, should be observed [13]. However, since myosin is not active in the presence of Cd^{2+}, an alternative approach was chosen to identify further the nature of the enzyme–product complex.

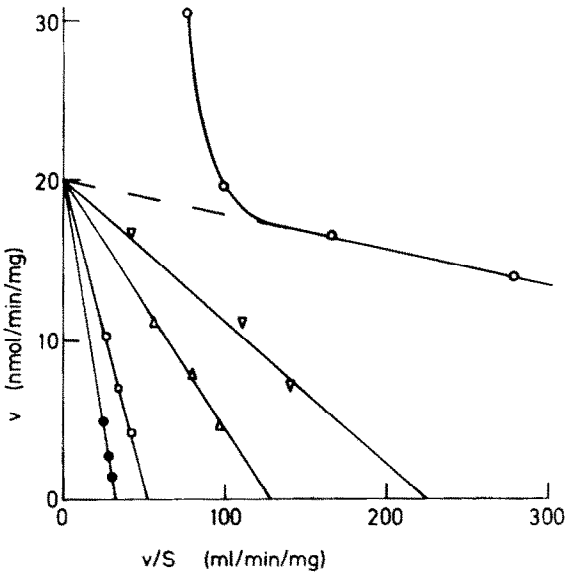

Fig.1. Eadie-Hofstee plot of myosin Mg^{2+}-ATPase (○- -○) and its inhibition by 10 μM ADP (●- -●), ADPβS (□- -□), ADPαS, A (△- -△), and ADPαS, B (▽- -▽). pH 8.0; other conditions are as in section 2.

The β-monodentate and αβ-bidentate complexes of CrADP were prepared and tested for their inhibitor activity against myosin Mg^{2+}-ATPase at pH 7.0. The following values were obtained: K_i (β-CrADP) = 3 μM and K_i (α,β-CrADP) = 101 μM (for ATP, K_m = 0.022 μM and V_{max} = 14 nmol . min^{-1} . mg^{-1}). If it is assumed that the αβ-CrADP consisted of a 50:50 mixture of the Δ and Λ diastereomers and that only one of the isomers is an inhibitor of myosin, then the β-monodentate chelate is still ~20-fold better inhibitor than

Table 1
Inhibition constants for ADP and its phosphorothioate analogs[a]

	Mg^{2+}-ATPase	Co^{2+}-ATPase
V_{max}, nmol . min^{-1} . mg^{-1}	20	26
K_m, μM	0.022	0.28
K_i (ADP), μM	0.39	1.1
K_i (ADPαS,A), μM	1.6	5
K_i (ADPαS,B), μM	3.5	9
K_i (ADPβS), μM	0.65	14

[a] Conditions are as in fig.1

either bidentate chelate. This result therefore confirms that the metal–ADP product exists as the β-monodentate chelate. (Compare with the results in [30], where no binding of α,β-bidentate CrADP to myosin subfragment-1 was observed.)

Dunaway-Mariano and Cleland [3] have classified kinases into two groups: those which bind ATP as a metal–α,β-bidentate complex (e.g., creatine kinase) and those wherein the β-monodentate metal–ADP complex is the product (e.g., yeast hexokinase). These authors found that most of the kinases so far studied belong in the former category whereas the few which belong in the latter category catalyze essentially irreversible reactions. Our results indicate that myosin belongs in the latter category, thus confirming and extending the generalization that enzymes which are specific for the β-monodentate metal–ADP chelate catalyze irreversible reactions.

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