Effect of Divalent Cations on the Formation and Stability of Myosin Subfragment 1−ADP−Phosphate Analog Complexes†

Y. Michael Peyser,‡ Michal Ben-Hur,‡ Moshe M. Werber,§ and Andras Muhlrad*,‡

Department of Oral Biology, Hebrew University-Hadassah School of Dental Medicine, Jerusalem 91120, Israel, and Bio-Technology General, Kiryat Weizmann, Rehovot 76236, Israel

Received October 27, 1995; Revised Manuscript Received January 10, 1996⊗

ABSTRACT: Myosin belongs to the family of motor proteins. Its interaction with actin coupled with hydrolysis of ATP is the molecular basis of muscle contraction. The head segment of myosin, called subfragment 1 (S1), contains the distinct binding sites for ATP and actin and responsible for the ATPase activity. The rate-limiting step of the ATP hydrolysis is the dissociation of the S1·MgADP·P_i complex which is accelerated by actin. The substitution of P_i with phosphate analogs (PA), such as vanadate (Vi) or beryllium fluoride (BeF_x), highly stabilizes the complex. We studied the role of the divalent cations in the ATPase activity and in the formation and decomposition of PA-containing stable complexes by substituting Mg^{2+} with Fe^{2+}, Mn^{2+}, Ni^{2+}, Co^{2+}, and Ca^{2+}. These metal ions supported the actin activation of S1 ATPase and affected the obtained kinetic parameters, K_M and V_{max}. The ATPase activity of S1 in the absence of actin increased with the increasing ionic radius of the metal (Me) ions. These ions also substituted for Mg^{2+} in the formation of the stable ternary S1·MeADP·PA complexes, which cannot be generated in the absence of divalent cations. Upon formation of stable ternary complexes, S1 reversibly loses its ability to catalyze the hydrolysis of ATP. The formation of the complexes can be followed by monitoring the disappearance of the ATPase activity. The rate of the complex formation depends on the divalent cation present and decreases in the order Mn > Fe > Ni > Co > Mg and Ca > Mn > Fe > Mg > Co in the Vi- and BeF_x-containing complexes, respectively. The ATPase activity of S1 is recovered upon addition of actin, which causes the decomposition of the complex. The spontaneous decomposition of the complexes was studied in the presence of ethylenediaminetetraacetic acid (EDTA), which chelates the metal divalent cations released from the complex and prevents its reformation. The rate of decomposition was assessed by monitoring the recovery of the ATPase activity of S1 in the presence of EDTA. The rate of decomposition of the Vi- and BeF_x-containing complexes follows the order Mn > Fe > Co > Mg > Ni and Ca ≫ Mn > Fe > Co > Mg, respectively. The rate of decomposition increases with the increasing ionic radius of the metal ions, similarly as observed in the case of ionic radius dependence of the ATPase activity. On the basis of this similarity, it is assumed that the decomposition of the complexes consists of two steps, the first step being the very slow release of PA followed by a rapid dissociation of MeADP from S1. The stability of the complexes has been calculated from the formation and decomposition rates. Except in the case of Mg, the stabilities of the BeF_x complexes are higher than those containing Vi. The results indicate that the metal cations have a significant role in maintaining the proper structure of the transient state complex in the myosin-catalyzed ATP hydrolysis.

Myosin, the main motor protein of muscle, and its active fragments catalyzes the hydrolysis of ATP essentially according to the following kinetic scheme (Taylor, 1979):

Scheme 1

$$M + ATP \overset{1}{\leftrightarrow} M^{*}\cdot ATP \overset{2}{\leftrightarrow} M^{**}\cdot ADP\cdot P_i \overset{3}{\leftrightarrow} M^{*}\cdot ADP + P_i \overset{4}{\leftrightarrow} M + ADP + P_i$$

M represents myosin or its active proteolytic fragments including myosin subfragment 1 (S1),[1] and one or two asterisks symbolize specific conformational changes taking place in the protein during the various stages of ATP hydrolysis. Since the dissociation of P_i in stage 3 is the rate-limiting step of the whole process, the M**·ADP·P_i transition state is the predominant intermediate of the myosin-catalyzed ATP hydrolysis. Actin, the other main structural protein of muscle, highly accelerates the release of P_i from the M**·ADP·P_i complex, and the dissociation of P_i is closely associated with the generation of force (power stroke) in the cross-bridge cycle during muscle contraction. All these point to the importance of the formation, decomposition, and structure of the M**·ADP·P_i complex in the understanding of the molecular events of muscle contraction. Under physiological conditions, the nucleotide is chelated with Mg^{2+} which highly contributes to the stability of the

† This research was supported by Grant 91-00027 from United States-Israel Binational Research Foundation (BSF), Jerusalem, Israel.

* Corresponding author: Andras Muhlrad, Department of Oral Biology, Hebrew University-Hadassah School of Dental Medicine, Jerusalem 91120, Israel.

‡ Hebrew University-Hadassah School of Dental Medicine.

§ Kiryat Weizmann.

⊗ Abstract published in *Advance ACS Abstracts,* March 1, 1996.

[1] Abbreviations: S1, myosin subfragment 1; S1Dc, truncated *Dictyostelium* S1; Me^{2+}, metal divalent cation; PA, phosphate analog; Vi, vanadate; BeF_x, beryllium fluoride complex; EDTA, ethylenediaminetetraacetic acid.

0006-2960/96/0435-4409$12.00/0

M**·ADP·P_i transient state (Bagshaw & Trentham, 1974). *In vitro* other divalent cations, including Ca^{2+}, Mn^{2+}, Co^{2+}, Ba^{2+}, Sr^{2+}, Fe^{2+}, Ni^{2+}, and Zn^{2+} are able to substitute for Mg^{2+} in the myosin-catalyzed ATP hydrolysis (Yount & Koshland, 1963; Seidel, 1969; Werber et al., 1973). Moreover, myosin also catalyzes the hydrolysis of ATP in the absence of divalent cations, provided certain monovalent cations are present. However, under these circumstances, the product release (steps 3 and 4) is much faster, while the ATP cleavage (step 2) is slower than in the presence of divalent cations (Lymn & Taylor, 1970). These results indicate that the nucleotide-attached metal ion has a substantial role in the myosin-catalyzed ATP hydrolysis.

Structural phosphate analogs, vanadate (Vi), beryllium fluoride (BeF_x), and aluminum fluoride (AlF_4) were found to substitute for P_i in the predominant M**·ADP·P_i transient state (Goodno, 1979; Phan & Reisler, 1992; Werber et al., 1992; Ponomorev et al., 1995). This substitution further stabilizes the complex and essentially "traps" the nucleotide together with the divalent cation at the active site in the protein (Goodno, 1979). These trapped complexes significantly contributed to the description of the atomic structure of S1 and particularly to the definition of the active site of myosin. Truncated *Dictyostelium* S1 (S1Dc), in complex with MgADP and BeF_x or AlF_4, was successfully crystallized, and its atomic structure was resolved (Fisher et al., 1995). The S1·MgADP·Vi complex was specifically oxidized and cleaved by vanadate-dependent photooxidation at Ser-180 and Ser-243, indicating the proximity of these residues to the phosphate binding loop in the active site of rabbit skeletal myosin (Cremo et al., 1989; Grammer & Yount, 1991; Muhlrad et al., 1991). Moreover, it became possible to covalently bind photoaffinity analogs of ATP to the protein following their trapping in the presence of divalent cations and phosphate analogs, which allowed the recognition of the specific purine and ribose binding loops (Cole & Yount, 1992; Yount et al., 1992; Luo et al., 1995) in the active site of myosin. Mg^{2+} could be replaced in the trapped complex by Ca^{2+}, Ni^{2+}, or Mn^{2+} (Grammer et al., 1988); however, no comprehensive study was carried out to reveal the contribution of divalent cations to the formation and stability of these complexes. In the present work, we studied the effect of various divalent cations, including Mg^{2+}, Mn^{2+}, Ca^{2+}, Ni^{2+}, Co^{2+}, and Fe^{2+}, on the ATPase activity of S1 in the presence and absence of actin and on the formation and decomposition of the BeF_x- and/or Vi-containing stable S1−ADP complexes. We found that all these cations can replace Mg^{2+} in the actin-activated S1 ATPase; however, they affect differently both V_{max} and K_M of the reaction (the latter indicates their differential influence on the S1−actin affinity in the presence of ATP). The presence of the cations was found to be an essential condition for the formation of trapped complexes. The various cations affected differently the formation and the rate of decomposition of the complexes. A correlation was found between the ionic radius of the metal ions, the ATPase activity in the absence of actin, and the decomposition of the complexes. A preliminary report of this work has been presented (Peyser et al., 1994).

MATERIALS AND METHODS

Chemicals. ATP, ADP, phenylmethanesulfonyl fluoride, dithioerythritol, chymotrypsin, sodium vanadate, $BeCl_2$ (dissolved in 1% HCl), HEPES, Tris-HCl, and the chloride salts of the divalent cations were purchased from Sigma Chemical Co. All other chemicals were of reagent grade. A stock solution of sodium vanadate (100 mM) was prepared according to Goodno (1979). Note that beryllium is very toxic and should be handled carefully. Fresh NaF and $FeCl_2$ stock solutions were prepared daily. To the $FeCl_2$ stock solution we added dithioerythritol in stoichiometric concentration in order to prevent the oxidation of Fe(II).

Preparation of Proteins. Myosin and actin were prepared from back and leg muscles of rabbit by the methods of Tonomura et al. (1966) and Spudich and Watt (1971), respectively. S1 was obtained by digestion of myosin filaments with chymotrypsin as described by Weeds and Taylor (1975). Protein concentrations were obtained by absorbance, using an A (1%) of 280 nm of 5.5 and 7.5 for myosin and S1 and A (1%) of 290 nm of 6.3 for actin, respectively. Molecular masses were assumed to be 500, 115, and 42 kDa for myosin, S1, and actin, respectively.

ATPase Assays. Actin-, Me^{2+}-, and K^+(EDTA)-activated ATPase activities (nanomoles of phosphate per micromole of S1 per minute) were calculated from the inorganic phosphate produced, measured according to Fiske and Subbarow (1925). The reaction was performed at 25 °C on 1 mL aliquots taken at various time intervals. Incubation times were chosen so that no more than 15% of the ATP was hydrolyzed. For actin-activated ATPase, the assay contained 0.2 μM S1, 0−40 μM F-actin, 1 mM chloride salt of the metal divalent cation, 2 mM ATP, 14 mM KCl, and 25 mM HEPES buffer (pH 7.0). The same solution without actin was used for measurement of the Me^{2+}-modulated ATPase activities. For K^+(EDTA)-activated ATPase, the reaction mixture contained 1 μM S1, 2 mM ATP, 6 mM EDTA, 600 mM KCl, and 50 mM Tris-HCl buffer (pH 8.0) and was incubated at 25 °C for 2 min.

Formation of Stable S1−Nucleotide Complexes. S1 (20 μM) was incubated in 30 mM HEPES (pH 7.0) at 25 °C with 0.2 mM ADP and with either 0.2 mM Vi or 0.2 mM $BeCl_2$ and 5 mM NaF for 20 min. After addition of 0−1.0 mM Me^{2+} (chloride salts), aliquots were taken at various time intervals to measure K^+(EDTA)-activated ATPase activity as described above. The apparent rate constant of complex formation, k_{obs}, was obtained from the rate of the ATPase activity loss.

Decomposition of the Stable S1−Nucleotide Complexes. This was studied by monitoring the dissociation of metal ions from the stable S1·MeADP·Vi and S1·MeADP·BeF_x complexes by EDTA chase. The released Me^{2+} was chelated with EDTA, which reacts only with free Me^{2+} but not with Me^{2+} trapped in the complex. The EDTA-chelated Me^{2+} cannot reenter the complex, which decomposes in the absence of divalent cation. The S1·MeADP·PA complexes were incubated at 25 °C in the presence of 4 mM EDTA, and the decomposition of the complex is followed by measuring the recovery of the K^+(EDTA)-activated ATPase activity as described above.

RESULTS

Effect of Divalent Metal Cations on the ATPase Activity of S1. The ATPase activity of S1 was measured at low ionic strength (pH 7.0) in the presence of 1 mM chloride salts of the various metal cations (Table 1). The activity depended on the nature of the divalent metal cation present and

Table 1: Kinetic Parameters of Actin-Activated ATPase

divalent cations	actin-activated ATPase			ATPase in the absence of actin (s^{-1})	actin activation[a]
	K_M (μM)	V_{max} (s^{-1})	V_{max}/K_M		
Mg	19.2	14.7	0.76	0.042	350
Fe	3.8	8.1	2.10	0.193	42
Co	7.3	10.8	1.48	0.152	71
Mn	12.4	16.4	1.33	0.203	81
Ni	31.8	3.6	0.11	0.0235	153
Ca	1.6	7.1	4.43	0.317	22

[a] V_{max} of the actin-activated ATPase/ATPase in the absence of actin. ATPase activity was assayed as described under Materials and Methods.

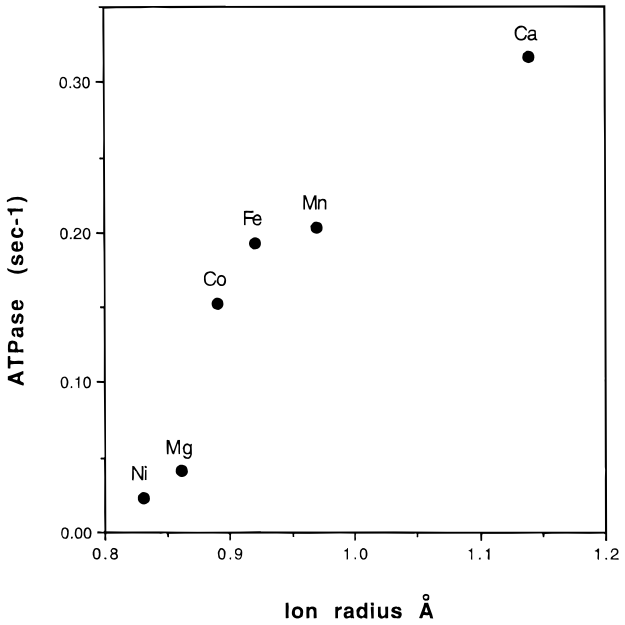


FIGURE 1: Relationship between ATPase activity in the absence of actin and the ionic radius of divalent metal cations. ATPase activities are taken from Table 1, and the ionic radius values are according to Cotton and Wilkinson (1988).

decreased in the order Ca > Mn > Fe > Co > Mg > Ni. The ATPase activity measured in the presence of various cations is well-correlated with the ionic radius of the cations (Figure 1). It increased with the ionic radius. Addition of increasing amounts of actin activated the ATPase activity of S1 in a saturable manner in the presence of all metal ions studied (Figure 2). For each metal ion, the K_M and V_{max} values were calculated from the dependence of the ATPase activity on actin concentration, using double reciprocal plots (Table 1). Both values were affected by the nature of the metal ion present. The affinity between F-actin and S1 in the presence of ATP, which is reflected by the inverted K_M values, decreased in the order Ca > Fe > Co > Mn > Mg > Ni. The maximal ATPase activity in the presence of actin, V_{max}, decreased in the order Mn > Mg > Co > Fe > Ca > Ni, which is quite different from the order obtained in the absence of actin. However, the efficiency of actin activation by the various metal ions is best characterized by division of the V_{max} values by the ATPase activities obtained in the absence of actin (Table 1). The most efficient cation of the actin activation is Mg, which is also the physiological cation of the actin-activated myosin ATPase, and the efficiency decreased in the order Mg > Ni > Mn > Co > Fe > Ca, showing the strong dependence of actin activation on the nature of the metal ion and emphasizing the fact that in the

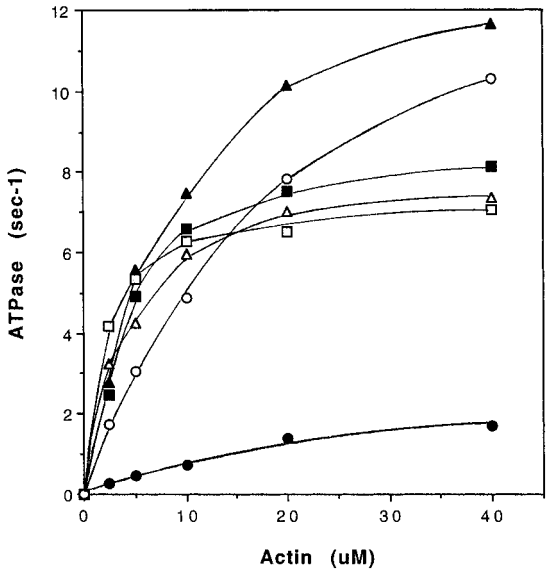


FIGURE 2: Effect of metal divalent cations on the actin-activated ATPase activity of S1. ATPase activity was assayed as described under Materials and Methods. Co (■), Mn (▲), Ni (●), Mg (○), Fe (△), Ca (□).

absence of actin the second lowest ATPase activity rate was obtained with Mg.

Formation of Stable S1·MeADP·PA Complexes. The formation of stable myosin−nucleotide complexes is accompanied by the loss of ATPase activity as it was shown for both the S1·MgADP·Vi (Goodno, 1979) and S1·MgADP·BeF_x complexes (Maruta et al., 1991; Werber et al., 1992; Phan & Reisler, 1992). We studied the role of various metal divalent cations in the formation of these complexes by monitoring the loss in the K^+(EDTA)-activated ATPase activity of S1 following its incubation with increasing amounts of metal ions in the presence of saturating concentrations of phosphate analogs (Vi or BeF_x) and ADP (Figure 3). No stable complex was formed in the absence of divalent metal cations. All the cations studied supported the formation of both complexes, with the exception of the S1·CaADP·Vi and S1·NiADP·BeF_x complexes, which according to the loss of ATPase activity did not form in a significant amount.

The kinetics of formation of the S1·MeADP·PA complexes was studied by monitoring the time course of loss of the K^+(EDTA)-activated ATPase activity of S1 in the presence of inhibitory concentrations of metal divalent cations at saturating ADP and PA concentrations. The time course of the loss of K^+(EDTA)-activated ATPase in the presence of 0.1 mM $MgCl_2$ and BeF_x is shown in Figure 4. The time course of complex formation in the presence of other metal cations was similar except for that with Mn^{2+} and Ca^{2+}, for which the loss of ATPase activity was too fast to monitor. The time courses of ATPase disappearance for all metal ions were fitted to a single exponential and characterized by a first-order kinetic constant, k_{obs}. The dependence of k_{obs} on the concentration of the various divalent cations in Vi- and BeF_x-containing complexes is shown in Figure 5. Hyperbolic curves were obtained in all cases which indicate that at high metal ion concentrations k_{obs} values approach saturation. This can be interpreted to mean that the formation of the S1·MeADP·PA complexes takes place at least in two steps; the first step is assumed to be a rapid binding equilibrium which is followed by a slow

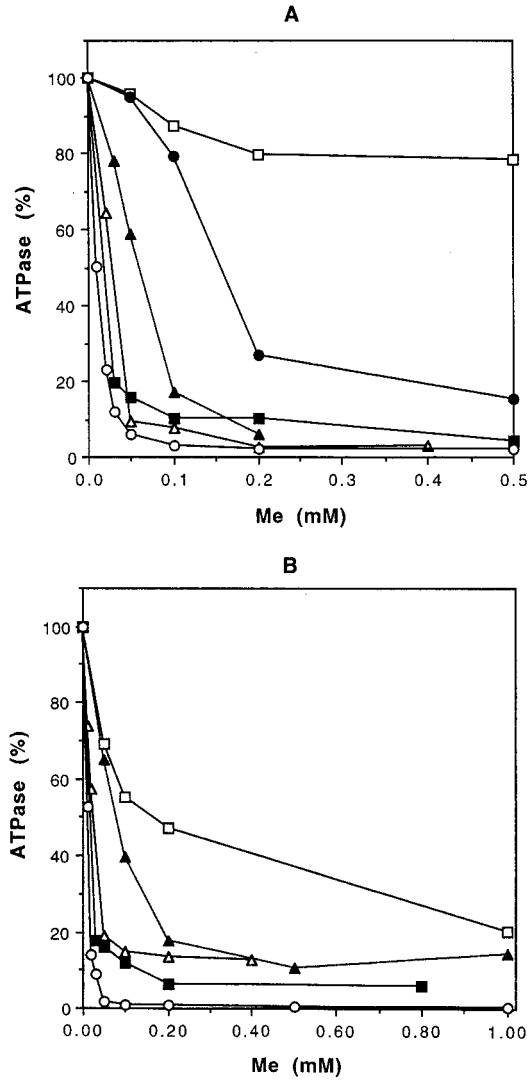


FIGURE 3: Relationship between loss of ATPase activity and divalent metal cation concentration during the formation of the stable S1·MeADP·PA complexes. S1 (20 μM) was preincubated at 25 °C for 5 min in 0.2 mM ADP and 30 mM HEPES (pH 7.0) with 0.2 mM Vi or 0.2 mM $BeCl_2$ and 5 mM NaF (in the case of the BeF_x-containing complex). After the preincubation, the chloride salts of metal ions were added to the reaction mixture and the incubation was continued for an additional 20 min. Following the incubation, aliquots were taken from the samples and their K^+-(EDTA)-activated ATPase activity was assayed as described under Materials and Methods. ATPase activity was expressed as percent of activity of S1 in the absence of phosphate analog: (A) Vi-containing and (B) BeF_x-containing complexes. Co (■), Mn (▲), Ni (●), Mg (○), Fe (△), Ca (□).

isomerization step, as proposed by us earlier (Werber et al., 1992) for the formation of the S1·MgADP·PA complexes, according to the following scheme:

Scheme 2

$$\text{S1} + \text{MeADP} + \text{PA} \underset{k_{-1}}{\overset{k_1}{\leftrightarrow}} \text{S1·MeADP·PA} \underset{k_{-2}}{\overset{k_2}{\leftrightarrow}} \text{S1\#·MeADP·PA}$$

S1# indicates an altered conformation. A similar mechanism for vanadate binding was suggested by Smith and Eisenberg (1990). The association constant of the first step, K_1, and the rate constant of the isomerization step, k_2, are obtained by plotting $1/k_{obs}$ against $1/[Me^{2+}]$ at saturating PA and ADP

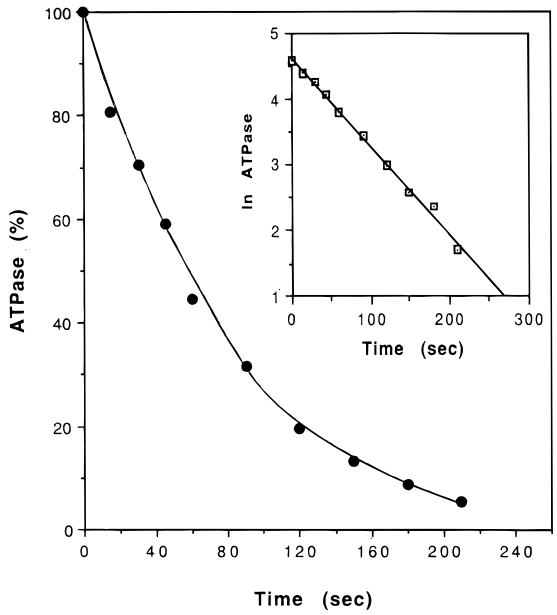


FIGURE 4: Time course of the formation of the S1·MgADP·BeF_x complex in the presence of 0.1 mM $MgCl_2$. The time course of the formation of the complex was followed by assaying K^+(EDTA)-activated ATPase activity of S1 as described under Materials and Methods. ATPase activity was expressed as percent of activity of S1 in the absence of phosphate analog: inset, natural log of ATPase activity plotted against time.

concentrations. The argument of the analysis, as developed by Werber et al. (1992), is the following. Scheme 2 is treated in a manner analogous to that of the reaction of an enzyme with a substrate. By plotting $1/V$ against 1/[Me], two steady state constants, $K_M = (k_2 + k_{-1})/k_1$ and $V_{max} = k_2$[S1·MeADP·PA], can be obtained. The rate constant of the dissociation of the stable complexes, k_{-2}, is small and, therefore, negligible, and k_2, the rate constant of the isomerization step, is assumed to be much smaller than k_{-1}. Therefore, K_M is equal to the dissociation constant of the nonstable intermediate S1·MeADP·PA and K_1, the association constant, equal to $1/K_M$. The kinetic parameters obtained according to this analysis are listed in Table 2. The rate of formation of the stable S1·MeADP·Vi and S1·MeADP·BeF_x complexes decreases in the order Mn > Fe > Ni > Co ≫ Mg and Ca ≈ Mn > Fe ≫ Mg > Co, respectively. Generally, the formation of Vi-containing complexes is faster than that of BeF_x-containing complexes with the exception of Mg, where the formation of the BeF_x-containing complex is faster. The value of the association constant, K_1, of the S1·MeADP·Vi and S1·MeADP·BeF_x complexes decreases in the order Mg > Co > Ni > Fe and Co > Mg > Fe, respectively.

Dissociation of Stable S1·MeADP·PA Complexes. The mechanistic basis of the actin activation of myosin ATPase is the dramatic acceleration of the dissociation of the M**·MgADP·P_i complex, which is the rate-limiting step of the myosin ATPase (Taylor, 1979). Similarly, the decomposition of the S1·MgADP·PA complexes is highly accelerated by actin (Goodno & Taylor, 1982; Werber et al., 1992; Phan et al., 1993). In this work, we observed that in addition to Mg other divalent metal cation-containing stable S1−nucleotide−PA complexes are dissociated by actin as indicated by the recovery of the K^+(EDTA)-activated ATPase activity upon addition of actin (Table 3). This finding indicates that all the stable S1·MgADP·PA complexes studied in the present

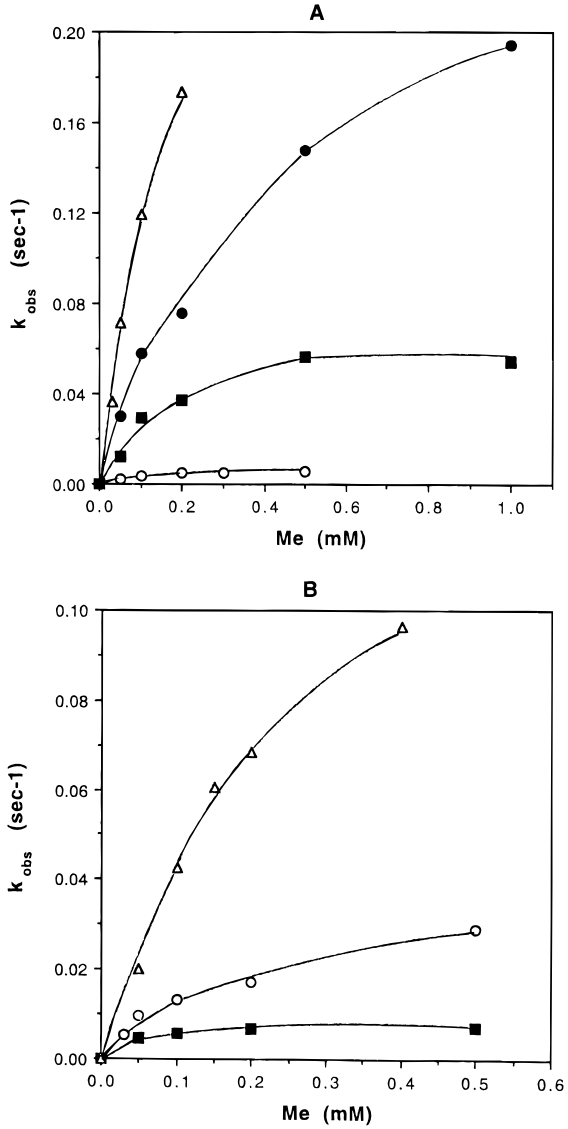


FIGURE 5: Dependence of the rate of formation of the stable S1·MeADP·PA complexes on the concentration of divalent metal cations. The time course of the formation of the complexes was followed by monitoring the loss of the K^+(EDTA)-activated ATPase activity as described under Materials and Methods, and the rate constants (k_{obs}) were calculated by fitting the obtained values to a single exponential: (A) Vi-containing and (B) BeF_x-containing complexes. Co (■), Ni (●), Mg (○), Fe (△).

work are good analogs of M**·MgADP·P_i and S1 remains native in the presence of these metals.

The spontaneous dissociation of the S1·MgADP·PA complexes was studied by EDTA chase which is detailed in Materials and Methods. The essence of this method is that EDTA chelates the metal cation which is released from the complex; because of the lack of free cation, the complex cannot reform, and therefore, the ATPase activity of S1 is restored. The time course of the spontaneous dissociation of the stable S1·MeADP·Vi and S1·MeADP·BeF_x complexes is followed by monitoring the recovery of the K^+(EDTA)-activated ATPase activity and presented in Figure 6. In the study of the dissociation of the S1·MgADP·BeF_x, S1·MgADP·Vi, and S1·NiADP·Vi complexes whose rates of decomposition are very slow, the reaction was followed for at least 2 days (results not shown). In all cases, except with S1·MgADP·BeF_x, the recovery has been steadily increased and hyperbolic time curves were obtained. The progress of

Table 2: Kinetic Parameters of the Formation and Dissociation of Stable S1−Nucleotide Complexes

divalent cation	k_2 (s^{-1})	k_{-2} (s^{-1})	K_1 (mM^{-1})	K_2	ionic radius[a] (Å)
Vi complex					
Mg	0.0067	1.64×10^{-5}	9.09	409	0.86
Fe	1.21	9.5×10^{-5}	1.07	12737	0.92
Co	0.11	3.5×10^{-5}	2.7	3142	0.89
Mn	*b*	56.6×10^{-5}	−	−	0.97
Ni	0.27	0.18×10^{-5}	2.17	152500	0.83
BeF_x complex					
Mg	0.043	0.145×10^{-5}	3.33	29655	0.86
Fe	0.52	95.6×10^{-5}	0.78	544	0.92
Co	0.015	37.5×10^{-5}	5.62	40	0.89
Mn	*b*	153.3×10^{-5}	−	−	0.97
Ca	*b*	2005×10^{-5}	−	−	1.14

[a] Ionic radius values of the divalent cations are taken from Cotton and Wilkinson (1988). [b] Too fast to measure. For calculations, see text.

Table 3: Complex formation in the Presence of Divalent Cations and Its Dissociation by Actin[a]

	ATPase activity in the percent of uncomplexed S1	
divalent cation	without actin	with actin
Vi complex		
no cation	99	100
Mg	4.1	98
Fe	4.5	99
Co	5.8	101
Mn	4.5	99
Ni	9.0	102
BeF_x complex		
no cation	92	98
Mg	6.9	101
Fe	14	99
Co	11.7	102
Mn	16	100
Ca	36	98

[a] S1 (20 μM) was incubated in 0.2 mM ADP, 30 mM HEPES (pH 7.0), and 0.2 mM Vi or 0.2 mM $BeCl_2$ and 5 mM NaF and with 0.2 mM divalent cation at 25 °C for 30 min. After the incubation, actin was added in a 2:1 molar ratio to S1, and the reaction mixture was further incubated for 30 min prior to measuring K^+(EDTA) ATPase.

the ATPase recovery with S1·MgADP·BeF_x has completely stopped after 12 h and remained at a constant level (8−9% of the activity obtained in the absence of the phosphate analog). This may be due to an additional isomerization step. A similar phenomenon was also observed by Phan and Reisler (1992). However, S1 also remained native in this complex, since its ATPase activity was completely recovered following addition of actin even after a 60 h incubation at 25 °C in the presence of EDTA. All the time courses of the ATPase recovery (with the exception of that of S1·MgADP·BeF_x, where only the initial ascending region was used) presented in Figure 6 were fitted to a single exponential and characterized by a first-order kinetic constant, k_{-2}, which is the rate constant of dissociation (Scheme 2). Since the rate constant of the isomerization step, k_2, is known from the experiments presented in Figure 5, the association constant, $K_2 = k_2/k_{-2}$, was calculated and is given together with the k_{-2} values in Table 2. The association constants, K_2, of the S1·MeADP·Vi and the S1·MeADP·BeF_x stable complexes decrease in the order Ni ≫ Fe > Co > Mg and Mg ≫ Fe > Co, respectively. The order of decrease of the rate constants of dissociation, k_{-2}, of the S1·MeADP·Vi and S1·MeADP·BeF_x complexes is Mn > Fe > Co > Mg > Ni and Ca > Mn > Fe > Co ≫ Mg, respectively. The rate of dissociation

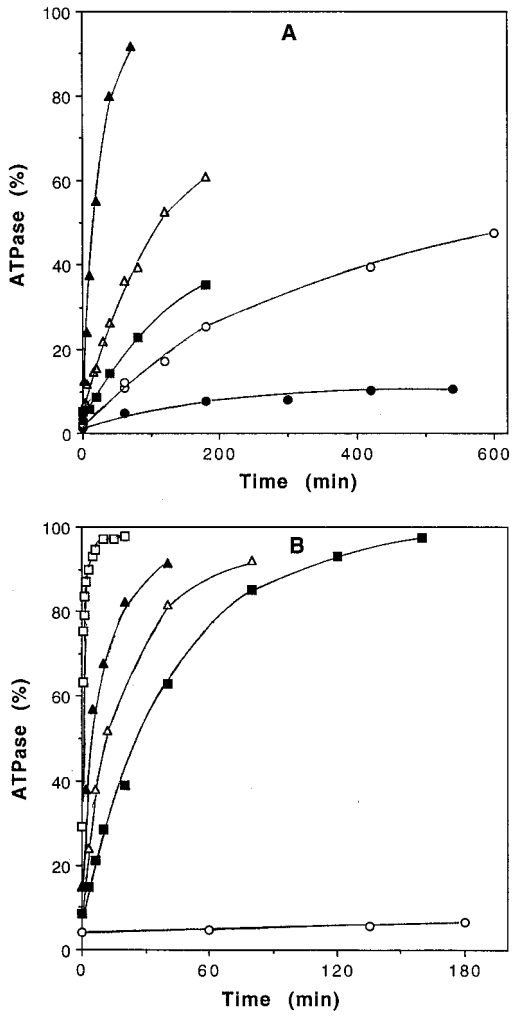


FIGURE 6: Effect of divalent metal cations on the decomposition of the S1·MeADP·PA complexes. The decomposition of the complexes was followed by monitoring the recovery of the K^+-(EDTA)-activated ATPase activity after addition of EDTA as described under Materials and Methods. ATPase activity was expressed as percent of activity of S1 in the absence of phosphate analog: (A) Vi-containing and (B) BeF_x-containing complexes. Co (■), Mn (▲), Ni (●), Mg (○), Fe (△), Ca (□).

of the S1·MeADP·BeF_x complexes is generally faster than that of the S1·MeADP·Vi complexes except in the case of Mg, where the opposite is true. A strong correlation was found between the ionic radius of the metal ions and the rate constant of dissociation, k_{-2}, of their respective complexes (Table 2). The rate of dissociation increases with the increase of the ionic radius. The ionic radius dependence of the rate of dissociation of the complexes is very similar to the ionic radius dependence of the ATPase activity measured in the presence of these cations in the absence of actin (Figure 1).

DISCUSSION

Under physiological conditions, myosin is a Mg^{2+}-dependent ATPase. Mg^{2+} binds together with ATP, in the form of a MgATP complex, to the active site of the enzyme. Mg cannot be exchanged during the course of ATP hydrolysis; it highly stabilizes the M**·MgADP·P_i transitions state and leaves myosin in the form of a MgADP complex at the end of the reaction (Bagshaw & Trentham, 1974; Mandelkow & Mandelkow, 1973). According to studies with sulfur-containing ATP model compounds (S replaces O in the α- and β-phosphates of the molecule in nonbridging position), Mg binds to the β- and γ-phosphates of ATP with the formation of a β,γ-bidentate complex (Goody et al., 1981; Connolly & Eckstein, 1981; Webb et al., 1982). The actual substrate is the Δβ,γ-MgATP isoform (Connolly & Eckstein, 1981). After the hydrolytic step (step 2 in Scheme 1), Mg remains attached to the β-phosphate of ADP as β-MgADP monodentate and is also coordinated to the inorganic phosphate (P_i) produced in the reaction, to water molecules, and to functional groups of myosin (Webb et al., 1982). According to the recently described atomic structure of the S1Dc·MgADP·BeF_x and S1Dc·MgADP·AlF_4 complexes (Fisher et al., 1995), Mg in *Dictyostelium* myosin is coordinated in an octahedral arrangement to the β-phosphate of ADP, the BeF_x or AlF_4 moiety, two molecules of water, and two protein ligands, Thr-186 and Ser-237. Other divalent metal cations are also supposed to bind to ATP, ADP, and myosin in a similar manner. They also stabilize the M**·MeADP·P_i transition state, making the dissociation of this complex the rate-limiting step of the reaction. This was proven for Mn by electron paramagnetic resonance (EPR) (Webb et al., 1982) and kinetic studies (Bagshaw, 1975) but also was indicated for Ca (Lymn & Taylor, 1970), Co, Zn, and Cd (Connolly & Eckstein, 1981).

In this work, we compared the effect of Mg^{2+}, Mn^{2+}, Co^{2+}, Fe^{2+}, Ni^{2+}, and Ca^{2+} on the ATPase activity of S1, on the actin activation of the ATPase activity, and on the formation and decomposition of Vi- or BeF_x-containing S1·MeADP complexes. All the metal divalent cations studied supported the actin activation. The degree of actin activation was a function of the nature of the metal ion present. The highest activation was obtained in the presence of Mg. The high efficiency of activation is very important energetically for the organism, since this enables very low energy consumption (ATP hydrolysis), during the relaxation of muscle when myosin heads are dissociated from actin and fast increase in ATP hydrolysis during force development in contraction. ATPase activity measured in the absence of actin was found to increase with the increasing ionic radius of the metals. These results are in agreement with findings reported earlier (Yount & Koshland, 1963; Seidel, 1969; Malik et al., 1972). No such correlation was observed for the ATPase activity measured in the presence of actin. This is probably due to the fact that, because of the accelerated phosphate release from the M**·MeADP·P_i complex, the dissociation of phosphate from the complex is not necessarily the rate-limiting step of the ATP hydrolysis in the presence of actin, and the new rate-limiting step may not be affected by the ionic radius of the metal ions. Moreover, the actin−myosin interaction is rather complex and is affected itself by the nature of the metal ion present. Therefore, a clear-cut correlation between actomyosin ATPase activity and ionic radius cannot be expected.

It was found in this work that the presence of divalent cations is a necessary requirement for the formation of stable S1·ADP·PA complexes and that the stability of the complexes depended on the nature of the metal divalent cation present. It is worthwhile to compare the phosphate analog-containing complexes with those obtained by cross-linking SH_1 and SH_2 thiols in the presence of nucleotides (Reisler et al., 1974; Wells & Yount, 1979; Wells et al., 1980), since also in the latter case nucleotide diphosphates were trapped only in the presence of divalent metal cations (Dalbey et

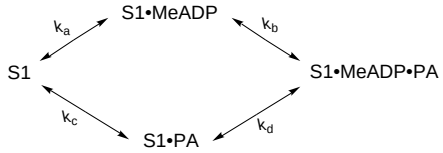
al., 1983). All the metal ions, Mg^{2+}, Mn^{2+}, Co^{2+}, Ni^{2+}, and Ca^{2+}, which promoted the trapping of ADP following the cross-linking of the SH_1 and SH_2 thiols were also found to promote the formation of the phosphate analog-containing complexes. This indicates the basic similarity of the two kinds of complexes and supports the hypothesis (Wells & Yount, 1979) that the distance of the two reactive thiols of myosin changes during the hydrolysis of ATP.

A rather stringent requirement, the inhibition of the K^+-(EDTA)-activated ATPase activity of S1, was used in our studies as an indicator of the stability of the S1·MeADP·PA complexes. This requirement is stringent because the high EDTA concentration, 6 mM, used in the ATPase assay practically reduces the free metal divalent ion concentration to zero and immediately chelates any metal ion released from the complex, thus preventing the reformation of the complex. Thus, we consider a complex stable only if it does not decompose during 2 min of incubation at 25 °C in the presence of EDTA. According to this criterion, Mg, Fe, Co, Mn, and Ni promote the formation of the S1·MeADP·Vi complexes, while Mg, Fe, Co, Mn, and Ca support the formation of the S1·MeADP·BeF_x complexes. The trapping of ADP and Vi in the presence of Mg, Mn, Co, and Ni was also shown by Grammer et al. (1988). On the other hand, according to our definition, Ca and Ni do not promote the formation of stable S1·CaADP·Vi and S1·NiADP·BeF_x complexes, respectively. The stringency of the criterion is supported by our recent finding that in the absence of EDTA the formation of the S1·CaADP·Vi complex is observed according to the near-UV circular dichroism spectrum (Ajtai, Peyser, and Muhlrad, unpublished results). The fact that a S1·CaADP·Vi complex, which is nonstable, can be formed at all indicates that the metal cation requirement for the formation of the complexes is less stringent than for its stability, which is essentially determined by the rate of decomposition of the complexes.

Since the formation of the complexes is accompanied by the loss of ATPase activity, it was important to test whether the heavy metal cations, like Fe^{2+}, which might react with the SH_1 thiol, do not denature S1 with irreversible loss of enzymatic activity. We did not find any denaturation, since upon addition of actin the K^+(EDTA)-activated ATPase activity of all complexes was completely recovered as it was observed earlier for both S1·MgADP·Vi and S1·MgADP·BeF_x complexes (Werber et al., 1992; Phan et al., 1993). The recovery of the K^+(EDTA)-activated ATPase activity strongly argues against the involvement of the SH_1 thiol in the reaction since it is well-known that the blocking of this thiol results in a severe inhibition of the K^+(EDTA)-activated ATPase activity of myosin.

The formation of the stable S1·MeADP·PA complexes is assumed to take place according to Scheme 2, which consists of a rapid equilibrium and a second slow isomerization step. The first step is quite complicated because there are four different reactants, S1, ADP, Me, and PA. According to the consensus theory, Me and ADP associates with S1 as a MeADP complex (Mandelkow & Mandelkow, 1973; Bagshaw & Trentham, 1974; Bagshaw, 1975; Webb et al., 1982). This decreases the number of reactants to three, and the formation of the nonstable intermediate is assumed to take place via two possible routes.

Scheme 3



It is difficult to decide whether $k_a \rightarrow k_b$ or $k_c \rightarrow k_d$ is the preferred route; however, there is evidence that both exist. The formation and dissociation of the S1·MeADP complex is well-documented [for review, see Taylor (1979)]. The existence of the S1·PA complex is indicated by the phosphate inhibition of the vanadate-dependent photocleavage at the active site of myosin (Muhlrad et al., 1991), by the strong static quenching of S1 tryptophan fluorescence by vanadate (Werber et al., 1992). Moreover, the atomic structure of the S1Dc·MgADP·BeF_x complex, showing that BeF_x is located at the back of the active site in a small pocket (Fisher et al., 1995), also indicates that P_i or phosphate analogs can enter more easily to the active site when its opening is not covered by the MgADP complex. However, the possibility that PA enters on the opposite side of the myosin head through a "back door" cannot be excluded (Yount et al., 1995).

Both the association constant for the formation of the nonstable intermediate, K_1, and the rate constant of the isomerization step, k_2, depend on the nature of the metal cation and phosphate analog present. The K_1 values do not correlate well with any known properties of the metal ions, including ligand preference, coordination geometry, or ionic radius, which may reflect on the complexity of the reaction described in Scheme 3. On the other hand, the k_2 values increse, with the exception of that of Co in the BeF_x- and Ni in the Vi-containing complexes, with the ionic radius of metal ions. In the case of Mg, both the K_1 and k_2 values are in agreement with our former results[2] obtained by monitoring tryptophan fluorescence intensity changes in S1 (Werber et al., 1992) and with those of Phan and Reisler (1992) assessed from the loss of ATPase activity during the formation of the S1·MgADP·BeF_x complex.

The rate constant of dissociation, k_{-2}, strongly correlates with the ionic radius; it increases with the ionic radius of metal ions. This observation is in agreement with those of Dalbey et al. (1983) obtained by monitoring the release of trapped ADP in the presence of different metal ions from SH_1-SH_2 cross-linked S1. The fact that both the rate of dissociation of the S1·MeADP·PA complexes and the steady state ATPase activity have a similar dependence on the ionic radius of metal ions indicates that the dissociation of the complexes and the rate-limiting step of the ATP hydrolysis, which is the dissociation of phosphate from the M**·MeADP·P_i transient state, follow a similar mechanism. On the basis of this similarity with the myosin-catalyzed ATP hydrolysis, we assume that during the decomposition of the stable complexes first the phosphate analog dissociates, which is the rate-limiting step, and this step is rapidly followed by the dissociation of MeADP:

Scheme 4

$$S1^{\#}\cdot MeADP\cdot PA \rightarrow S1\cdot MeADP + PA \rightarrow S1 + MeADP$$

[2] There are calculation errors in Table 1 of our former paper (Werber et al., 1992). The correct values of K_M (μM) and k_2 (s^{-1}) are as follows: S1−MgADP−BeF_3^-, 589 and 65 × 10^{-3}; S1−MgADP−AlF_4^-, 133 and 4 × 10^{-3}; S1−MgADP−Vi, 80 and 8 × 10^{-3}.

The identical ionic radius dependence of the dissociation of the M**·MeADP·P_i and $S1^{\#}$·MeADP·PA complexes also supports the widely held view (Goodno, 1979; Werber et al., 1992; Phan & Reisler, 1992; Phan et al., 1993) that the PA-containing stable complexes are good analogs of the predominant transient state of the myosin-catalyzed ATP hydrolysis. The ionic radius dependence of the stability of the complexes is related to the tertiary structure of the myosin active site. According to Fisher et al. (1995), BeF_x binds to a small pocket at the bottom of the active site and is covered by ADP which occupies the upper part of the active site. The metal cation is coordinated to the β-phosphate of ADP, the BeF_x moiety, two water molecules, and two functional groups of the protein. There is a tight stereochemical fit, which the ionic radius of the metal ionic should satisfy. The best fit can be obtained with Mg, which is the physiological ion of the myosin ATPase. Metal ions with higher ionic radii can achieve only a looser stereochemical fit, which leads to a faster release of the phosphate analog through the back door of the active site (Yount et al., 1995). As a consequence of the nonperfect fit, a change occurs in the tertiary structure of the motor domain of S1, and it probably also affects the conformational changes accompanying the release of phosphate or phosphate analogs. In addition to the ionic radius, there are other parameters, like charge and coordination geometry, which may affect the metal ion dependence of the stable complexes. The charge or charge density seems to be a very important factor, since monovalent cations with similar ionic radii but about half of the charge density of the divalent cations used in this study do not support the formation of stable complexes. Another possible factor is the coordination geometry. All the metal ions used in this work have preferred octahedral (or tetrahedral geometry with the possibility also of being octahedral) with the exception of Ni, which in some cases prefers square planar geometry. Ni is an exception also in our study, since it does not form a stable S1·NiADP·BeF_x complex. It is quite possible that the reason for the exceptional behavior of Ni is its preference for planar geometry, which means that it may bind to less protein ligands, hence in some cases forms less stable complexes than other metal ions.

In conclusion, we found that a number of divalent metal cations can substitute for Mg, the physiological cation, in actin activation of myosin ATPase. The parameters of the ATPase activity both in the presence and in the absence of actin are significantly affected by the nature of the metal ion added. Stable S1·ADP·PA complexes are formed only in the presence of divalent cations. The stability of the complexes is essentially determined by the rate of dissociation of the phosphate analog from the complexes. This rate as well as the ATPase activity in the absence of actin are strongly correlated with the ionic radius of the metal ion present, which implies that the dissociation of the phosphate analog is the rate-limiting step in the decomposition of the PA-containing stable S1−nucleotide complexes. The results indicate that the metal ions significantly affect the structure of the stable complexes and, in analogy, also the structure of the predominant intermediate of the myosin-catalyzed ATP hydrolysis.

REFERENCES

Bagshaw, C. R. (1975) *FEBS Lett. 58*, 197−201.

Bagshaw, C. R., & Trentham, D. R. (1974) *Biochem. J. 141*, 331−349.

Cole, D. G., & Yount, R. G. (1992) *Biochemistry 31*, 6186−6192.

Connolly, B. A., & Eckstein, F. (1981) *J. Biol. Chem. 256*, 9450−9456.

Cotton, F. A., & Wilkinson, G. (1988) *Advanced Inorganic Chemistry*, 5th ed., pp 1385−1388, John Wiley & Sons, New York.

Cremo, C. R., Grammer, J. C., & Yount, R. G. (1989) *J. Biol. Chem. 264*, 6608−6611.

Dalbey, R. E., Wells, J. A., & Yount, R. G. (1983) *Biochemistry 22*, 490−496.

Fisher, A. J., Smith, C. A., Thoden, J. B., Smith, R., Sutoh, K., Holden, H. M., & Rayment, I. (1995) *Biochemistry 34*, 8960−8972.

Fiske, C. H., & Subbarow, Y. (1925) *J. Biol. Chem. 66*, 375−400.

Goodno, C. C. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 2620−2624.

Goodno, C. C., & Taylor, E. W. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 21−25.

Goody, R. S., Hofmann, W., & Konrad, M. (1981) *FEBS Lett. 129*, 169−172.

Grammer, J. C., & Yount, R. G. (1991) *Biophys. J. 59*, 226a.

Grammer, J. C., Cremo, C. R., & Yount, R. G. (1988) *Biochemistry 27*, 8408−8415.

Luo, Y., Wang, D., Cremo, C. R., Pate, E., Cooke, R., & Yount, R. G. (1995) *Biochemistry 34*, 1978−1987.

Lymn, R. W., & Taylor, E. W. (1970) *Biochemistry 9*, 2975−2983.

Malik, M. N., Marchioli, L., & Martonosi, A. (1972) *Arch. Biochem. Biophys. 153*, 147−154.

Mandelkow, E. M., & Mandelkow, E. (1973) *FEBS Lett. 33*, 161−166.

Maruta, S., Henry, G. D., Sykes, B. D., & Ikebe, M. (1991) *Biophys. J. 59*, 436a.

Muhlrad, A., Peyser, Y. M., & Ringel, I. (1991) *Biochemistry 30*, 958−965.

Peyser, Y. M., Werber, M. M., & Muhlrad, A. (1994) *Biophys. J. 66*, A75.

Phan, B., & Reisler, E. (1992) *Biochemistry 31*, 4787−4793.

Phan, B. C., Faller, L. D., & Reisler, E. (1993) *Biochemistry 32*, 7712−7719.

Ponomarev, M. A., Timofeev, V. P., & Levitsky, D. I. (1995) *FEBS Lett. 371*, 261−263.

Reisler, E., Burke, M., Himmelfarb, S., & Harrington, W. (1974) *Biochemistry 13*, 3837−3840.

Seidel, J. C. (1969) *Biophys. Biochim. Acta 189*, 162−170.

Smith, S. J., & Eisenberg, E. (1990) *Eur. J. Biochem. 193*, 69−73.

Spudich, J. A., & Watt, S. (1971) *J. Biol. Chem. 246*, 4866−4871.

Taylor, E. W. (1979) *CRC Crit. Rev. Biochem. 6*, 103−114.

Tonomura, Y., Appel, P., & Morales, M. F. (1966) *Biochemistry 5*, 515−521.

Webb, M. R., Ash, D. E., Leyh, T. S., Trentham, D. R., & Reed, G. H. (1982) *J. Biol. Chem. 257*, 3068−3072.

Weeds, A. G., & Taylor, R. S. (1975) *Nature (London) 257*, 54−56.

Wells, J. A., & Yount, R. G. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 4966−4970.

Wells, J. A., Knoeber, C., Sheldon, M. C., Werber, M. M., & Yount, R. G. (1980) *J. Biol. Chem. 255*, 11135−11140.

Werber, M. M., Szent-Gyorgyi, A. G., & Fasman, G. D. (1973) *J. Mechanochem. Cell Motil. 2*, 35−43.

Werber, M. M., Peyser, Y. M., & Muhlrad, A. (1992) *Biochemistry 31*, 7190−7197.

Yount, R. G., & Koshland, D. E. (1963) *J. Biol. Chem. 238*, 1708−1713.

Yount, R. G., Cremo, C., Grammer, J. C., & Kerwin, B. A. (1992) *Philos. Trans. R. Soc. London, Ser. B 336*, 55−61.

Yount, R. G., Lawson, J. D., & Rayment, I. (1995) *Biophys. J. 68*, 44s−47s.

BI952565R