Chemomechanical Transduction in the Actomyosin Molecular Motor by 2′,3′-Dideoxydidehydro-ATP and Characterization of Its Interaction with Myosin Subfragment 1 in the Presence and Absence of Actin†

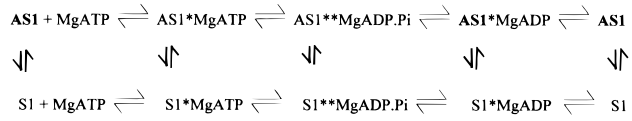
Damodaragounder Gopal,‡ Dimitrii I. Pavlov,§ Dimitrii I. Levitsky,§ Mitsuo Ikebe,|| and Morris Burke*,‡

Department of Biology, College of Arts and Sciences, Case Western Reserve University, Cleveland, Ohio 44106, A. N. Bach Institute of Biochemistry, Russian Academy of Sciences, Moscow 11701, and the Department of Physiology, University of Massachusetts Medical Center, Worcester, Massachusetts 01655

Received March 11, 1996; Revised Manuscript Received June 6, 1996⊗

ABSTRACT: The effect of torsional freedom about the *N*-glycoside bond of ATP in the ability of the nucleoside triphosphate to support chemomechanical transduction (Takenaka et al., 1978) has been investigated by examining the ability of the nucleotide analogue 2′,3′-dideoxy-2′,3′-didehydro-ATP (**1b,** enf-ATP) to act as a substrate for myosin subfragment 1 in the presence and absence of actin and to support actin sliding in the standard *in vitro* motility assay. By converting the ribosyl ring of the natural substrate to the rigid and almost planar enofuranosyl ring, effects on torsional freedom about the *N*-glycoside bond due to changes in ribosyl ring pucker and/or by steric interferences of the protons attached to the 2′ and 3′ carbons are eliminated allowing for increased torsional freedom about the *N*-glycoside bond. The data indicate that this enofuranosyl analogue is an excellent substrate for subfragment 1 and actosubfragment 1 and produces actin sliding velocities which are twice as fast as those observed with ATP in the standard *in vitro* motility assay. The analogue diphosphate is trapped in S1 by the common P_i analogues, but the rate of formation of the ternary complex formed with V_i is very slow compared to that observed with MgADP. Similar conformations of S1 are formed with Mg·enf-ATP and MgATP under steady-state conditions, but S1 with bound Mg·enf-ADP differs significantly from that observed with MgADP.

One of the major questions concerning the molecular mechanism of muscle contraction and other actomyosin based motor functions is the conformational role of the fuel molecule in the energy coupling process (Takenaka et al., 1978). While many natural and synthetic nucleoside triphosphates are capable of acting as substrates for myosin and actomyosin, the ability of these to support chemomechanical transduction appears to be very selective, indicating that key interactions with the protein involving the heterocyclic base are necessary for force or contraction to occur (Tonomura, 1973). It is well established that actomyosin based contractility and motility arise by conformational changes in this protein complex occurring as a direct consequence of the MgATPase hydrolysis cycle. The hydrolysis of MgATP by myosin S1 in the absence and presence of actin has been well characterized [reviewed in Taylor (1979), Cooke (1986), Goldman (1987), Homsher and Millar (1990)] and can be represented by the following simplified scheme:



where the bold AS1 denotes strongly actin bound states of S1.

In the absence of actin, the rate-limiting step is the isomerization associated with the transition from the S1**MgADP·P_i[1] state to the S1*MgADP state (Bagshaw & Trentham, 1974), concomitant with or preceding the release of P_i from the protein. In the presence of actin, the rate-limiting step is either bond hydrolysis (Rosenfield & Taylor, 1984) or product release (Lymn & Taylor, 1971), but force is thought to be associated with the transition from the non force-producing attached state, A·S1**MgADP·P_i, to the force-producing state, A·S1*MgADP state. Although the rate constants of the individual steps may vary, all analogues which act as substrates must proceed through this basic cycle, whether or not they support energy coupling. It appears, from these considerations, that the requirement of an analogue to support contraction is its ability to form a state, analogous to the S1**MgADP·P_i, which in the absence of actin is rate limiting, and which, in its presence, is significantly slower than the rate of actin reassociation with it. Only under these conditions can a significant fraction of the S1 cross bridges attach to actin and proceed through the force-generating step in the MgATPase cycle and, therefore, support energy coupling.

† This work was supported by U.S. Public Health Service Grant NS-15314 (M.B.), and a Fogarty Grant 1 RO3 TW00270-01 (D.I.L).

* Author to whom correspondence should be addressed.
‡ Department of Biology, Case Western Reserve University.
§ Russian Academy of Sciences.
|| Department of Physiology, University of Massachusetts.

⊗ Abstract published in *Advance ACS Abstracts,* July 15, 1996.

[1] Abbreviations: S1, myosin subfragment 1; S1Dc, motor domain of *Dictyostelium discoideum* S1; S1**MgADP·P_i and S1*MgADP, myosin subfragment 1 states under steady-state conditions and with bound MgADP, respectively, with the number of asterisks indicating the degree of S1 tryptophyl fluorescence; enf-ATP and enf·ADP are the triphosphate and diphosphate of 2′,3′-dideoxy-2′,3′-didehydroadenosine, respectively; dATP, 2′-deoxy-ATP; NEM, *N*-ethyl maleimide; Tris, tris(hydroxymethyl)aminomethane; V_i, orthovanadate; BeF_x, beryllium fluoride; AlF_4^-, tetrafluoroaluminate; ScF_x, fluoroscandium anion; DSC, differential-scanning calorimetry.

S0006-2960(96)00597-1 CCC: $12.00

Of the naturally occurring nucleoside triphosphates, only CTP is capable of supporting contraction to an extent comparable to ATP (Pate et al., 1993; Regnier et al., 1993). Since both compounds have amino groups at equivalent positions, C4 for CTP and C6 for ATP, it appears that their similar chemomechanical efficiency may be related to the ability of this amino group to act as a donor to form a specific hydrogen bond with the protein (Pate et al., 1993). The recent structure of S1Dc containing trapped MgADP indicates that such an interaction exists and that the acceptor group is the phenolic oxygen of the conserved Tyr-135 (Fisher et al., 1995). In this connection, it was previously shown that the binding of ATP, ADP, or PP_i to HMM perturbed the UV absorption spectra of S1 in a way consistent with perturbation of a tyrosyl residue at or near the active site (Morita & Yagi, 1966; Sekiya & Tonomura, 1967) with the effect being markedly enhanced under steady-state conditions. Furthermore, because of the marked enhancement of the S1 tryptophyl fluorescence, it is also likely that this interaction, while P_i is still bound at the active site, is also responsible for the marked S1 tryptophyl fluorescence enhancement observed with these two nucleotides (Werber et al., 1972). Thus, the effectiveness of the natural nucleoside triphosphates to support contraction must be intimately related to its ability to "stabilize" a non-force-producing state of S1 following bond hydrolysis, and this appears to be dependent on the heterocyclic base moiety.

An additional factor that may be needed for effective energy coupling by the actomyosin motor is the degree of torsional freedom of the *N*-glycosidic bond. This possibility was raised from the observations that C8-substituted adenosine triphosphates, constrained to the syn conformation, and C8-C3′- and C8-C2′-S-cyclo derivatives of ATP, which were locked in anti conformations, were unable to support contraction despite the fact they could be effectively hydrolyzed by myosin (Takenaka et al., 1978). To address this question, we have synthesized 2′,3′-enofuranosyl ATP and examined its ability to act as a substrate for S1 and actoS1 and to support actomyosin motility. This analogue was chosen since the main consequence of the enofuranosyl substitution for the ribosyl ring would be an enhanced rotational freedom about the *N*-glycosidic bond due to the planar nature of this ring and the sp2 hybridization at the 2′ and 3′ carbons. The data show that this analogue produces actin sliding velocities over immobilized myosin which are twice those observed in the presence of MgATP. Functionally, enf-ATP is an excellent substrate for S1 and actoS1 yielding turnover rates comparable to those observed with MgATP. The data suggest that, under steady-state conditions with Mg•enf-ATP, S1 adopts a conformation similar to the S1**MgADP•P_i state observed with the natural substrate, but the binding of the diphosphate form of the analogue produces an altered conformation of the protein leading to a much slower rate of trapping by V_i than that occurring with MgADP. An examination of the crystallographic structures of adenosine (Lai & Marsh, 1972), 2′,3′-dideoxydidehydroadenosine (Hutcheon & James, 1974), and those of MgADP trapped in S1 by BeF_x and AlF_4- (Fisher et al., 1995) reveals that the main change of the enofuranosyl substitution is in the torsional angle, about the C1′−C4′ virtual bond since the distance between the C5′ and the N9 atoms across the enofuranosyl ring is only slightly reduced.

MATERIALS AND METHODS

Reagents. ATP, ADP, DTT, Tris, KF, $POCl_3$ dimethylformamide, methanol, trimethyl phosphate, 2,6-lutidine, tributyl amine, triethyl amine, pyrophosphoric acid, DEAE Sephadex A-25, *N*-ethyl maleimide, sodium orthovanadate, NaI, $BeCl_2$, $AlCl_3$, and chymotrypsin were from Sigma and Aldrich. $ScCl_3$ was purchased from Johnson Mathey Electronics. All other reagents were analytical grade.

Proteins. Skeletal muscle myosin was prepared from the back and thigh muscles of New Zealand white rabbits by the procedure of Godfrey and Harrington (1972). S1 and actin were prepared from the rabbit skeletal muscle as described by Weeds and Taylor (1975) and by Spudich and Watts (1971), respectively.

Synthesis of 2′,3′-Dideoxy-2′,3′-didehydro-ATP (**1b**). 2′,3′-Dideoxy-2′,3′-didehydroadenosine (**1a**) was synthesized as reported by Krawczyk and Townsend (1989). 2′,3′-dideoxy-2′,3′-didehydro-ATP (**1b**) was synthesized using the procedure of Kovacs and Otvos (1988) and Goody and Isakov (1986). The enofuranosyl adenosine (**1a**, 466 mg) was added to a solution of phosphorus oxychloride (2 equiv) in trimethylphosphate (12 mL) at 4 °C in the presence of 2,6-lutidine (2 mL) and stirred for 15 min. A solution of tributylammonium salt of pyrophosphate prepared from pyrophosphoric acid (2.1 g) and tributylamine (4.2 g) was dissolved in dimethylformamide (10 mL) to which tributylamine (8.1 mL) was added. The pyrophosphate solution in DMF (distilled over phosphorus pentoxide) with excess tributylamine was cooled to 4 °C and added to a vigorously stirring phosphorylation mixture. After being stirred 1 min at 4 °C, the reaction mixture was poured in to a solution of 30 mL of triethylammonium bicarbonate (1 M, pH 7.6) and crushed ice (10 g). Excess tributylamine was extracted with 150 mL of diethyl ether. The products can be checked by TLC (silica) using *n*-propanol, aqueous ammonia, and water (11:7:2). The aqueous solution containing the required triphosphate was separated using the Sephadex A-25 column (45 cm length and 2.5 cm diameter). A linear gradient of 0−1 M triethyl ammonium bicarbonate buffer, pH 7.6, was used to elute the triphosphate. A total volume of 6 L of the buffers was used with a flow rate of 2.5 mL/min. The fractions (25 mL) 114−125 were evaporated at room temperature and dissolved in dry methanol (this process repeated three times) to isolate the triethyl ammonium salt of enofuranosyl adenosine as a oily residue which was converted to the sodium salt (Yount et al., 1971). The sodium salt of enf-ATP was dried over phosphorus pentoxide in a desiccator (yield 31%). The purity of the compound was confirmed by TLC giving a single spot and by NMR (in D_2O with pyridine-d_5) yielding a spectrum essentially identical to that reported for enf.adenosine (**1a**) (Krawczyk & Townsend, 1989). The isolated sodium salt was analyzed for the phosphate content confirming the triphosphate structure (Haley & Yount, 1972). A solution of this compound in water was found to be unstable but was stable in slightly alkaline conditions, and it was prepared as a stock solution (100 mM) in Tris buffer (0.5 M, pH 8.0). The concentration of the stock solution were determined by UV (259 nm, ϵ 16 465 M^{-1}) in Tris buffer (0.05 M, pH 8.0). This extinction coefficient is based on the value determined for **1a**. The dry solid stored air-tight at −20 °C was stable,

and the stock solution stored at −20 °C can be used for up to 2 weeks.

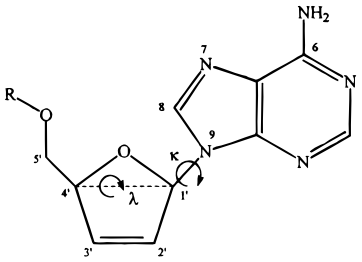
ATPase Assays. These were done by the procedures of Kielley and Bradley (1956), Yamaguchi and Sekine (1966), and White (1982) for the EDTA/K^+, Ca^{2+}, and actin activated Mg^{2+} ATPases, respectively. The actin activated Mg•enf-ATPase and MgATPase were assayed in 1.0 mM KCl, 50 mM imidazole, 3 mM $MgCl_2$, and 3 mM triphosphates, pH 7.0. The lower KCl concentration was used to increase the affinity of S1 for actin in the presence of the analogue. The S1 concentration was 0.05 mg/mL, and the actin concentrations ranged from 0.5 to 4.0 mg/mL. The amount of phosphate formed was measured spectrophotometrically by the method of White (1982). The actin activated data were plotted as both direct and double-reciprocal plots and the V_{max} and K_{app} for actin binding obtained by curve fitting the data using SlideWrite Plus (Advanced Graphics Software).

In Vitro Motility Assay. This was done by the procedure of Toyoshima et al. (1987). The movement of actin was observed upon addition of ATP or NTP with a fluorescence microscope and recorded on video tape. Speeds of actin displacement were analyzed by replay of the video tapes.

Fluorescence Measurements. These were done on a Perkin Elmer LS-5B spectrofluorometer at 20 °C. A solution of S1 (0.9 −1 μM) in 1 mM $MgCl_2$, 30 mM Tris-HCl, 0.04 mM enf-ATP, and 10 mM NaF, pH 7.9 (adjusted at room temperature), was kept at 20 °C for 30 min to allow the hydrolysis of the triphosphate to diphosphate. The hydrolysis of enofuranosyl triphosphate was followed by monitoring the decrease in the intrinsic fluorescence of the tryptophan (excitation at 295 nm and the emission at 335.5 nm). To the resulting mixture, the phosphate analogues (BeF_x, AlF_4^- and ScF_x) were added to a final concentration of 0.5 mM. The excitation wavelength was 295 nm, and the emission spectra was monitored at 335.5 nm.

Trapping of Enofuranosyl Diphosphate with V_i and BeF_x. Trapping by the phosphate analogues was done by incubating S1 (0.5 mg/mL) at 25 °C in 0.05 M Tris (pH 7.9, adjusted at room temperature, solvent A), 10 mM KF (for BeF_x), and 0.2 mM enofuranosyl triphosphate in the presence of 1.0 mM $MgCl_2$. After 1 h the P_i analogues (V_i and Be^{2+}) are added to a final concentration of 1 mM. Aliquots were removed and immediately assayed for ATPase activity. The rate of inhibition was also studied in the same buffer in the presence of 1.0 mM ATP by following the rates of P_i formation as described by White (1982).

NEM Modification of S1. S1 (8.7 μM) in KCl (0.05M) buffered with Tris-HCl (0.05 M) to pH 7.9. Prior to modification, $MgCl_2$ (1 mM) and enf-ATP (0.5 mM) were added, and the mixture was allowed to incubate for 2 h at 25 °C to complete the hydrolysis of enf-ATP to enf-ADP. NEM (4 mol/mol of S1) was then added, and, at desired times, aliquots were removed and diluted in KCl, Tris buffer, containing 1 mM in DTT to terminate the reaction. The diluted aliquots were subsequently assayed for Ca^{2+} ATPase in 0.5 M KCl at 37 °C using the procedure of Yamaguchi and Sekine (1965). To examine the dependence of the inactivation rate by NEM modification on the concentration of Mg•enf-ADP, the same basic protocol was used except that the preincubations with Mg.•enf-ATP were done at concentrations between 25 and 500 μM. The EDTA/K^+ ATPase activity of the modified samples was subsequently measured by the procedure of Kielley and Bradley (1956).



1

a: R = H
b: R = Triphosphate
c: R = Diphosphate

FIGURE 1: Structure of 2′,3′-enofuranosyl adenosine triphosphate showing the torsional angles κ and λ for the "glycosidic" N9−C1′ bond and the virtual bond C1′−C4′, respectively.

Table 1: S1 and actoS1 ATPase and enf-ATPase (s^{-1})

assay system	ATPase	enf-ATPase
NH_4^+/EDTA	27.2 ± 0.06	24.1 ± 0.12
Ca^{2+}	3.0 ± 0.06	4.1 ± 0.10
Mg^{2+}	0.052 ± 0.002	0.128 ± 0.002
Mg^{2+} (actin)	9.4 ± 1.3	16.0 ± 1.5
	(69 ± 10 μM)[a]	(106 ± 22 μM)[a]

[a] Values in parenthesis are the K_{app} for actin binding.

Differential Scanning Microcalorimetry (DSC). Calorimetric measurements were done on a differential adiabetic scanning microcalorimeter DASM-4 (Biopribor, Puschino, Russia) as described previously (Levitsky et al., 1992). The heating rate was 1 K/min. Prior to DSC experiments, enf-ATP (0.2 mM) was incubated overnight with S1 (1.5 mg/mL) at 4 °C in 30 mM Hepes, 1mM $MgCl_2$, at pH 7.3, to convert the enf-ATP to enf-ADP. Ternary complex formation with V_i was initiated by addition of V_i to 0.2 or 0.6 mM and incubating for 30 min at 20 °C after which the samples were stored on ice. For the complexes formed with BeF_x, the S1 and the Mg•enf-ADP was heated to 20 °C and upon addition of NaF to 5 mM was incubated for 5 min at this temperature. $BeCl_2$ was added to give final concentrations of 0.2 and 0.4 mM and the reaction mixtures were allowed to incubate at 20 °C for an additional 25 min before placing on ice. The DSC experiments were done on the same day. Control samples with ADP were obtained in the same way, but ADP (0.2 mM) was simply added to the S1. Experiments to monitor the stability of these complexes were done measuring the DSC of samples after 24 h dialysis versus 30 mM Hepes, 1.0 mM Mg Cl_2 at 4 °C.

RESULTS

In Vitro Motility and ATPase Properties of enf-ATP. To determine whether enf-ATP (**1b**, Figure 1) could support chemomechanical transduction in the actomyosin system, the standard *in vitro* motility assay was used to examine the ability of this analogue, to support actin sliding over immobilized myosin. The data revealed that actin sliding velocities were twice as fast as those observed with ATP [9.3 (±1.1) and 4.5 (±0.86) μm/s, respectively], indicating that enf-ATP has higher chemomechanical efficiency than ATP for the actomyosin motility system. The S1 enf-ATPase properties are presented in Table 1 together with those observed for ATP. It is clear that with respect to these properties enf-ATP is quite similar to those of the natural

substrate with hydrolysis in the presence of EDTA/K^+ 6−8 times higher than that occurring in the presence of Ca^{2+}. The effect of actin on the hydrolysis of enf-ATP by S1 in the presence of Mg^{2+} is also presented in Table 1. The maximum velocity at infinite actin concentration (16.0 s^{-1}) and the K_{app} (106 μM) for the analogue are about 2 and 1.5 times those observed with ATP, respectively. Therefore, these data indicate that enf-ATP is an excellent substrate for S1 and actoS1 and that it supports motility very well.

S1 Tryptophyl Fluorescence in the Presence of enf-ATP and enf-ADP. To obtain information about the nature of the steady-state conformation of S1 in the presence of Mg·enf-ATP, the S1 intrinsic tryptophyl fluorescence of S1 in the presence and absence of this analogue was next measured. The extent of enhancement observed with the former (27.0%) is similar to that observed with MgATP (29.5%) and suggests that a structure comparable to S1**MgADP·P_i is attained by the protein with this analogue under steady-state conditions. Upon hydrolysis of the analogue to the diphosphate form, the S1 emission spectra becomes essentially indistinguishable from that for S1 in the absence of nucleotide. Although this could imply that the analogue diphosphate does not bind to S1, chemical modification studies (discussed below) indicate that this diphosphate binds with essentially the same affinity to S1 as MgADP. The inability of the analogue diphosphate to increase the intensity of the tryptophyl fluorescence, therefore, points to an altered interaction with the protein compared to the situation occurring when MgADP is bound. These data indicate that under steady-state conditions with this analogue the rate-determining step is the isomerization associated with P_i release from the protein.

enf-ADP Binding Affects the Reactivity of the ATPase Related Thiols SH1 (Cys-707) and SH2 (Cys-697). It is well documented that communication exists between the nucleotide binding site and the 11 residue segment containing the SH1 (Cys-707) and SH2 (Cys-697). For example, the reactivities of these thiols to *N*-substituted maleimidyl reagents is extremely sensitive to the occupancy of the active site and to the nature of the nucleotide bound, and, moreover, modifications at SH1 or SH2 with NEM bring about characteristic changes in the ATPase properties of the protein (Yamaguchi & Sekine, 1966; Reisler et al., 1974). When the site is vacant or when occupied by PP_i, only SH1 shows high reactivity for these reagents causing characteristic changes to the ATPase properties upon modification. In the presence of MgATP, where the pre-dominant species under steady-state conditions is S1**MgADP·P_i, with both products present in the active site, both SH1 and SH2 are relatively unreactive. Release of P_i from the S1 active site, and the accompanying isomerization into a force-inducing conformation, results in an increase in reactivity to NEM of both thiols and their modification results in a loss of ATPase function. It was of interest, therefore, to examine whether a similar pattern would be observed with enf-ATP and enf-ADP. Figure 2 shows the rate of inactivation of S1 EDTA/K^+ ATPase induced by reaction with NEM in the presence of either Mg·enf-ATP or Mg·enf-ADP. It is evident that, under steady-state conditions in the presence of Mg·enf-ATP, there is a significantly lower inactivation rate than is observed when Mg·enf-ADP is bound at the active site. This protective effect by the analogue triphosphate is comparable to the situation observed for modification in the presence of

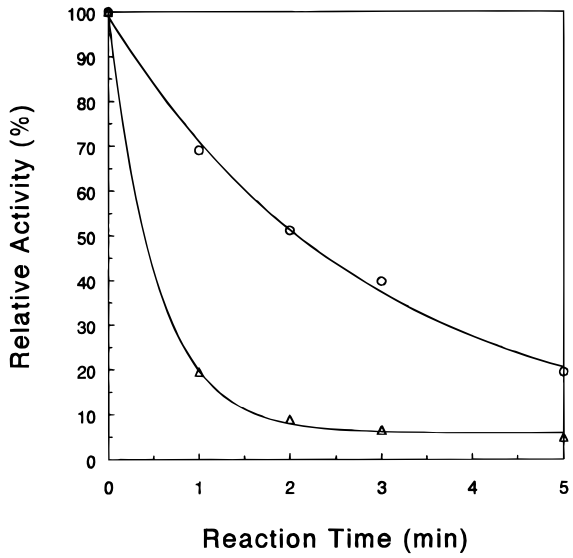


FIGURE 2: Inactivation of S1 EDTA/K^+ ATPase by modification of S1 with NEM in the presence of (○) Mg·enf-ATP and (△) Mg·enf-ADP·S1 (8.7 μM) in KCl (0.05 M), Tris·HCl (0.05 M), pH 7.9. was modified with a 4 M excess of NEM as described in Materials and Methods.

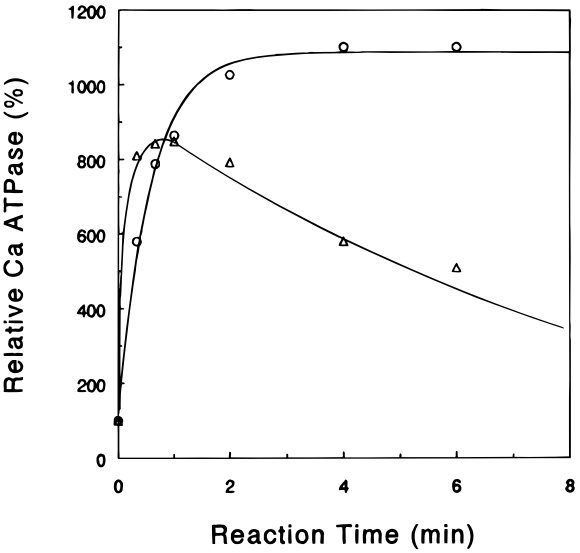


FIGURE 3: Effect of NEM modification of S1 in the presence of MgADP (△) and Mg·enf-ADP (○) on Ca^{2+} ATPase activities of the protein. Conditions used were the same as described in the legend to Figure 2.

MgATP and suggests that the conformation of the steady-state intermediate formed with this analogue is quite similar to that formed with MgATP. If it is assumed that the inactivation observed in the presence of Mg·enf-ATP reflects the amount of S1 in the S1·Mg·enf-ADP state under steady-state conditions, then the amount of S1 in the protected state (assumed to be the S1**Mg·enf-ADP·P_i state) can be estimated by extrapolation of the initial slopes of the inactivation data as shown in Figure 2, yielding a value of about 80% under steady-state conditions.

The time course of changes in the Ca^{2+} ATPase properties of S1 produced by NEM modification in the presence of Mg·enf-ADP or of MgADP is shown in Figure 3. In the case of modification in the presence of the latter, the activity is initially enhanced and then declines and has been previously shown to be associated with modification at both SH1 and SH2 (Reisler et al., 1974). For modification in the

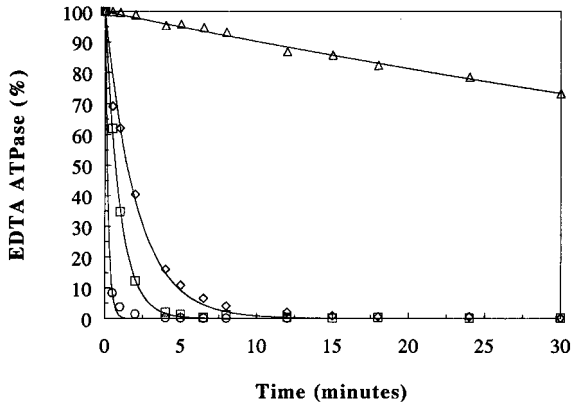


FIGURE 4: Rate of ternary complex formation for S1 in the presence of Mg·enf-ADP with (△) V_i and (◇) BeF_x and in the presence of MgADP with (□) V_i and (○) BeF_x. Conditions were as described in Materials and Methods.

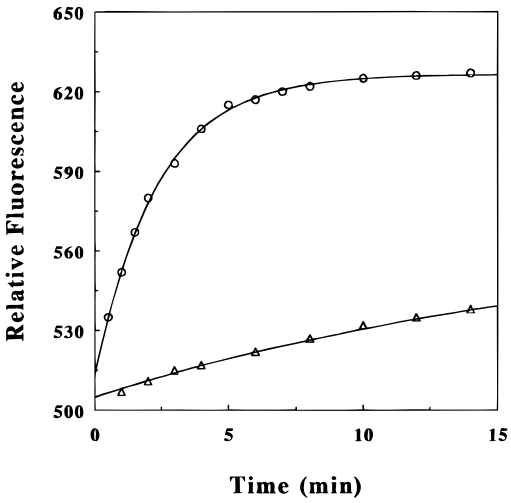


FIGURE 5: Rate of S1·Mg·enf-ADP tryptophyl fluorescence enhancement upon addition of ScF_x (○) and ALF_4^- (△).

presence of Mg·enf-ADP, this activity is markedly enhanced and does not decline in the time interval examined. This pattern of activation is indicative of modification at the SH1 thiol only, since modification at SH2 alone by NEM results in only slight activation of the S1 Ca^{2+}ATPase (Reisler et al., 1974). Therefore, Mg·enf-ADP binding to the S1 active site causes a perturbation in the thiol-containing region, but, unlike MgADP, the analogue does not appear to enhance the reactivity of SH2 to NEM.

As indicated earlier, the lack of an effect by Mg·enf-ADP on the S1 tryptophyl fluorescence could be indicative of poor binding of this analogue diphosphate to S1. Since the data presented in Figures 2 and 3 indicate that the reactivity of SH1 is enhanced in the presence of Mg·enf-ADP, an estimate of the binding affinity was made by measuring the inactivation rates of S1 EDTA/K^+ ATPase by NEM modification as a function Mg·enf-ADP concentration. These experiments indicated that the S1 (8.7 μM) is essentially fully complexed in the presence of 25 μM Mg·enf-ADP (data not shown) which would yield a binding constant of at least 1×10^5 M^{-1}, comparable to that noted for MgADP (Greene & Eisenberg, 1980).

Trapping of Mg·enf-ADP in S1 in the Presence of Various P_i Analogues. A good correlation exists between the effectiveness of nucleoside and non-nucleosides triphosphate analogues to support actomyosin based contraction and motility and the ability of their diphosphate forms to be trapped in S1 by a variety of P_i analogies such as V_i, BeF_x, and AlF_4^- (Bobkov & Levitsky, 1995). Therefore, since Mg·enf-ATP supports actomyosin-based motility, it was expected that Mg·enf-ADP would be readily trapped in S1 by these P_i analogues resulting in the inactivation of the S1 ATPase. This was first examined for the P_i analogues V_i and BeF_x by determining the inactivation rate of S1 EDTA/K^+ ATPase on incubating S1 with Mg·enf-ADP in the presence of a number of these P_i analogues, since formation of stable S1 ternary complexes with Mg·enf-ADP should result in an inactivation of this ATPase. The results are presented in Figure 4 together with data obtained using MgADP as a control. It is evident that for both of these P_i analogues, the rates of inactivation with Mg·enf-ADP are much slower than those observed with MgADP, especially in the case where V_i was used. The apparent first-order rate constant for inactivation by MgADP with V_i was 1.7×10^{-2} s^{-1} whereas it was 1.7×10^{-4} s^{-1} when Mg·enf-ADP was used, yielding apparent second order rate constants for ternary complex formation of 17 and 0.17 M^{-1} s^{-1}, respectively. This rate difference of two orders of magnitude was unexpected and cannot be attributed to poor binding by the analogue diphosphate based on the results of the Mg·enf-ADP concentration dependence of the rate of inactivation by NEM. A lower rate of trapping was also observed with Mg·enf-ADP when BeF_x was used to form the ternary complexes, compared to that observed with MgADP, but the difference here was only about 10-fold; the apparent first order rate constants being 8.0×10^{-3} and 8.0×10^{-2} s^{-1}, respectively.

The formation rates of the ternary complex were also followed by determining the rates of S1 tryptophyl fluorescence enhancement observed at 25 °C when P_i analogues were added to S1 in the presence of Mg·enf-ADP, since this enhancement induced by addition of the P_i analogue is attributable to ternary complex formation. A typical result is shown for trapping by ScF_x and AlF_4^- in Figure 5, yielding apparent first order rates of 7.1×10^{-3} and 8.3×10^{-4} s^{-1}, respectively. The first order rate constant for S1·Mg·enf-ADP·BeF_x formation by this method was found to be 5.3×10^{-3} s^{-1}, corresponding to a second order rate constant of 10.6 M^{-1} s^{-1} in reasonable agreement with a value of 8.1 M^{-1} s^{-1} estimated from the inactivation data (Figure 5). Thus, all the P_i analogues tested were able to trap Mg·enf-ADP in S1, but the formation rates were found to be extremely slow, especially in the case of V_i, in comparison to formation rates observed with MgADP.

DSC Analyses of the Binding of Mg·enf-ADP to S1 in the Absence and Presence of P_i Analogues. DSC analyses of S1 in the presence and absence of nucleotide have provided clear evidence of changes in the domain stability of S1 associated with different nucleotide bound states (Levitsky et al., 1992; Bobkov et al., 1993; Bobkov & Levitsky, 1995). This technique was used in the present study to examine the effect of binding of Mg·enf-ADP to S1, in the absence and presence of various P_i analogues, on the thermal stability of the protein. Figure 6 shows the DSC scans of S1 incubated with Mg·enf-ADP in the absence and presence of V_i. In this case, the incubations with V_i were done for 30 min at 20 °C, and the samples placed on ice prior to initiating the DSC scans. Two distinct transitions can be observed for the samples incubated with V_i. The first transition has a T_m

Table 2: Molecular Dimensions of Free Adenosine, Enofuranosyl Adenosine, and S1 Bound Forms of MgADP

angle or distance	adenosine[a]	enofuranosyl adenosine[b]	S1·MgADP· AlF_4^-	S1·MgADP· BeF_x
C8−N9−C1′−O4′ (χ)	(9.9°)[c]	(72.7°)[c]	−13.9°	18.7°
N9−C5′	4.1 Å	3.9 Å	4.2 Å	4.1 Å
N9−C1′−C4′−C5′ (λ)	25.4°	−3.7°	18.7°	16.6°
O−P(β)−O			132.7°	107.9°
P(α)−O−P(β)			134.4°	131.5°

[a] Lai and Marsh (1982). [b] Hutcheon and James (1974). [c] Values in parentheses represent N9−C1′ torsional angles in crystals of the nucleosides.

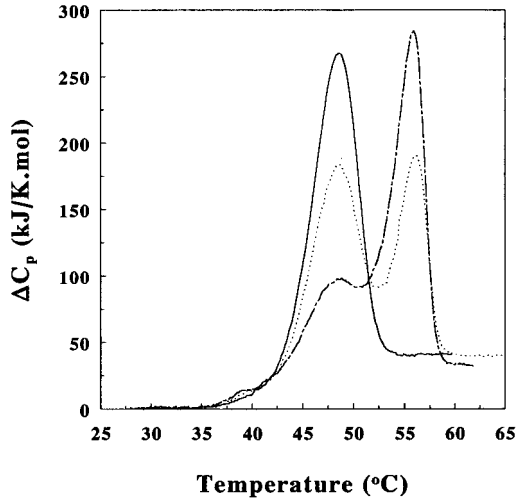


FIGURE 6: Temperature dependence of excess heat capacity for S1·Mg·enf-ADP incubated in the absence of V_i (—) and in the presence of 0.2 mM V_i (···) and 0.6 mM V_i (— · —).

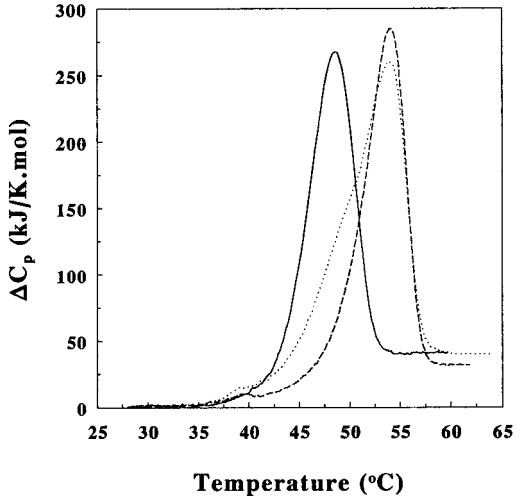


FIGURE 7: Temperature dependence of excess heat capacity for S1·Mg·enf-ADP in the absence (—) and presence of 0.2 mM (···) and 0.4 mM (---) BeF_x.

of ~47 °C similar to that observed for S1 in the presence of this analogue when V_i is omitted from the mixture, and, therefore, it can be attributed to the binary complex S1·Mg·enf-ADP. The second transition, with a T_m of approximately 56 °C, can be attributed to the presence of the stable ternary complex, S1·Mg·enf-ADP·V_i. This T_m is only slightly lower than that observed for the ternary complex formed with MgADP (58 °C; Bobkov & Levitsky, 1995), indicating that these complexes are likely to have similar conformations. Under the conditions used here, the amount of ternary complex present in the mixture corresponds to just under 50%, and reflects the slow rate of ternary complex formation with this analogue and V_i in accord with the inactivation data presented earlier (Figure 4). Incubations with higher molar amounts of V_i (0.6 mM) showed a significantly higher conversion to the ternary complex under the same incubation conditions.

The DSC scans for S1 with Mg·enf-ADP in the absence and presence of BeF_x are shown in Figure 7. It is evident that BeF_x also causes formation of a ternary complex, since the DSC scan shows an asymmetric peak due to the presence of a second transition with a T_m of approximately 53 °C and that the extent of ternary complex formation is dependent on the concentration of BeF_x present in the incubation mixture.

The stability of these S1 ternary complexes formed with Mg·enf-ADP was examined after dialysis at 4 °C for 24 h by DSC, and the resulting DSC profiles were compared to those obtained for S1 ternary complexes formed in a similar manner with MgADP. In contrast to the complexes formed with MgADP, those formed with Mg·enf-ADP have significantly lower stability, since the DSC scans indicate more than 50% of the complex has decayed by this overnight dialysis at 4 °C.

The effect of deoxygenation at the 2′ or 3′ position, or at both of these sites, of ADP on the ability to from ternary complexes with V_i and BeF_x was also investigated by DSC. In all these cases, rapid and stable trapping was observed in a manner indistinguishable from that observed with MgADP (Pavlov and Levitsky, unpublished results). It appears, therefore, that simple removal of the hydroxyl groups from the 2′ and 3′ positions of the ribosyl of adenosine diphosphate does not compromise the ability of these deoxy and dideoxy analogue diphosphates to be effectively trapped.

Conformational Analyses of Free Adenosine and Enofuranosyl Adenosine and of MgADP Bound in S1. The crystallographic structures of free adenosine and enofuranosyl adenosine are known, and the changes induced in the backbone structure associated with these different rings have been compared (Lai & Marsh, 1972; Hutcheon & James, 1974). Table 2 provides information about the conformations of these free nucleosides. While the torsional angles (χ) about the N9−C1′ (glycoside) bond for these free nucleosides are substantially different, these differences most likely reflect influences of intermolecular interactions within the crystal lattice and are likely to be different for the free nucleotides and for the nucleoside tri- and di- phosphates when bound to proteins. On the other hand, the distance between the N9 and C5′ atoms and the torsional angle (λ) about the C1′−C4′ virtual bond are intrinsic properties of the enofuranosyl adenosine and will not be changed by phosphorylation or by the binding of this analogue to S1.

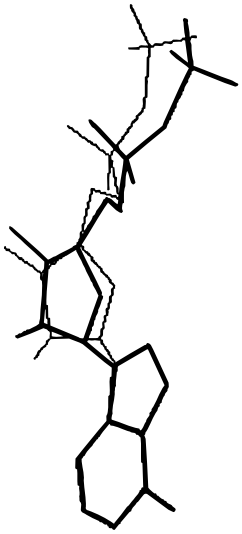


FIGURE 8: Structures of S1 bound MgADP in the ternary complexes formed with S1Dc and BeF_x (thick bonds) and AlF_4^-. The purine rings have been overlapped to show the deviations in the conformations in these two states.

However, in the case of adenosine, these two structural parameters are expected to be sensitive to changes in the ring pucker and, most likely, to the phosphorylation state of the nucleoside in free and protein-bound states. While the distance between N9 and C5′ is decreased slightly upon introduction of the double bond between C2′ and C3′, the torsional angle λ associated with the virtual C1′−C4′ bond changes by about 30°.

The recent solution of the crystallographic S1 structures with MgADP trapped by either BeF_x or AlF_4^- enables a comparison of the structure of the nucleotide as it is processed through the stage formed upon binding to the protein to the transition state leading to terminal bond hydrolysis. Some of the structural parameters associated with these S1 bound forms of MgADP were measured from coordinates kindly provided by Dr. Ivan Rayment and are also given in Table 2. Of particular interest is the findings that the structure of the bound MgADP is different in these two complexes. In particular, there is a significant difference in the torsional angle of the *N*-glycosidic bond (χ) of about 33°, and the O−P(β)−O angles in these complexes differ by almost 25°. Additional differences between these MgADP conformations are apparent in Figure 8, where the adenine rings of these bound forms of MgADP have been overlaid to enhance the comparison.

DISCUSSION

The major findings of this study are that enf-ATP is an excellent substrate for S1 and actoS1, and that it produces actin sliding velocities over immobilized myosin which are twice as fast in the standard *in vitro* motility assay as those observed when MgATP is used. This enhanced chemomechanical efficiency must, therefore, be related in some fashion to the introduction of the double bond between the 2′ and 3′ carbon atoms of the ribosyl ring, and, therefore, the structural consequences of this modification and how it may alter the manner in which adenosine triphosphate interacts with myosin and actomyosin must now be addressed. The first point to be considered is the structural changes imposed on the nucleotide by the enofuranosyl ring substitution. The presence of the double bond freezes the structure of the analogue between N9 and C5′ atoms, and this is independent of its phosphorylation state and of whether it is bound to the protein. Moreover, since the enofuranosyl ring is essentially planar (Hutcheon & James, 1974), it cannot undergo the usual puckering associated with the ribosyl ring. The two hydroxyl groups associated with the 2′ and 3′ carbons are also lost, but it appears that these groups are not involved in any interaction with the protein and are solvent exposed (Fisher et al., 1995), and, therefore, it is unlikely that they play a decisive role in the ability of ATP to support contraction.

The first conclusion, therefore, that can be drawn from this study is that flexibility in the region between the triphosphate chain and the adenine ring is not crucial for energy coupling, which suggests that the main function of the ribosyl ring is to act as a spacer and to constrain the steric relationships of these moieties within narrow limits, as originally proposed by Tonomura (1973). Furthermore, the two ribosyl hydroxyl groups lost in the formation of the enofuranosyl ring are also not required for contraction. This would be consistent with the observation that non-nucleoside analogues such as NANTP, which have an amino ethyl linker instead of a ribosyl ring, can also support contraction (Nakamaye et al., 1985).

The second point for consideration is to what extent does the ring substitution affect other structural parameters associated with the normal substrate. A number of the more pertinent structural values of enf-adenosine and adenosine are given in Table 2 (Lai & Marsh, 1972; Hutcheon & James, 1974), together with the corresponding distances for MgADP bound in the *Dictyostelium discoideum* S1 motor domain in the ternary complexes formed with BeF_x and AlF_4^- (Fisher et al., 1995). The distance between the N9 and C5′ atoms ranges from 4.1 to 4.2 Å for the normal ribosyl adenosine analogues, whereas it is lower in enofuranosyl adenosine (3.9 Å). The torsional angle, λ, of the C1′−C4′ virtual bond has very similar values for the two S1 bound forms of MgADP (16.6° and 18.7°) and is somewhat greater in free adenosine (25.4°), but it is significantly different for enofuranosyl adenosine (−3.7°). However, an examination of molecular models indicates that these distortions of the structure between N9 and C5′, produced by the enofuranosyl substitution, would not significantly alter the steric disposition of the triphosphate chain relative to the adenine ring, especially given the torsional freedom at the C4′-C5′ and *N*-glycoside bonds. Therefore, we would anticipate that Mg·enf-ATP would bind to S1 in a similar fashion to MgATP and produce similar structural changes in S1 upon hydrolysis of the terminal phosphoryl group. The S1 tryptophyl fluorescence measurements and the chemical modification data appear to support these expectations. Under steady-state conditions Mg·enf-ATP produces an enhancement of the S1 tryptophyl fluorescence close to that observed with MgATP, indicating that it forms a structure similar to the S1**MgADP·P_i conformation. Moreover, the protection afforded the reactive thiols SH1 and SH2 to modification by NEM by this conformational state with MgATP (Harrington et al., 1975) is also observed with Mg·enf-ATP (Figure 2).

Despite the apparent conformational similarities of the steady-state intermediates formed by Mg·enf-ATP and MgATP with S1, the stabilities of these complexes are significantly different. This is apparent in the turnover rates

(Table 1) indicating that P_i is released from the ternary product complex, S1**Mg·enf-ADP· P_i, faster than from the S1**MgADP·P_i complex. While the actual hydrolysis rate of Mg·enf-ATP by S1 remains to be determined, it is clear from the fluorescence and chemical modification data, that P_i release from the active site is also rate limiting for this analogue in the absence of actin.

Another property which is affected by the enofuranosyl substitution in ATP and ADP is the torsional freedom about the *N*-glycosidic and the C4′−C5′ bonds. The sp2 character of the 2′ and 3′ carbons places their attached hydrogens in the same plane as the enofuranosyl ring and, as a result, decreases their ability to interfere with rotation about these bonds due to steric effects (Hutcheon & James, 1974). The possibility that rotation about the *N*-glycosidic bond is crucial for energy coupling by the actomyosin motor was raised by Takenaka et al. (1978), and the enf-ATP analogue was synthesized to test the corollary of this proposal: increased torsional freedom should lead to enhanced energy transduction in the actomyosin motor. Since it appears that "freezing" the structure between the N9 and the C5′ atoms produces minimal displacements of the adenine ring and the triphosphate groups relative to the situation with ATP, no significant alterations in the ability of this analogue and MgATP to interact with S1 through these moieties would be expected. Furthermore, it appeared unlikely from the X-ray data of S1 with trapped MgADP that the double bond of the enofuranosyl ring interacts with the protein (Fisher et al., 1995). In this connection, the work by Shimizu et al. (1991) provide some insight into the role of the 2′- and 3′-hydroxyl groups of ATP in actomyosin chemomechanical transduction. With 2′- or 3′-deoxy-ATP they observed actin sliding velocities of 87% and 89%, respectively, of the value observed with ATP, while with dideoxy-ATP they observed values of about 104%. It would appear from these observations that the 2′- and 3′-hydroxyls of the ribosyl ring play little if any role in chemomechanical transduction as measured by actin filament velocities. However, in single fibers, 2′-deoxy ATP was found to produce maximum shortening speeds that were 144% of those observed with ATP (Regnier et al., 1993) suggesting that the ribosyl hydroxyls may have some influence in energy transduction. It is entirely possible that the puckering of the furanosyl ring may be affected by removal of one or both hydroxyls, but in either case the ring could not be planar, since the deoxy- and dideoxy-ATP analogues have a single bond between the 2′ and 3′ carbons. Therefore, the most plausible explanation for the enhanced chemomechanical efficiency of Mg·enf-ATP appears to be the enhanced rotational freedom associated with the *N*-"glycosidic" bond resulting from the enofuranosyl ring substitution. This conclusion is not meant to imply that other factors, including specific interactions of the adenine ring with the protein, are unimportant for energy coupling. Clearly, certain specific interactions are required between the base moiety and the protein to produce energy coupling. For example, despite the fact that all the naturally occurring nucleoside triphosphates would be expected to have similar torsional freedom over much of the allowable anti range available for both the purines and pyrimidine bases, only CTP promotes contraction comparable to MgATP. Thus, their 4-amino and 6-amino groups, respectively, are likely to play a key role in energy transduction by interacting with the protein (Tonomura, 1973; Pate et al., 1993) as suggested from the recent crystallographic data where the 6-amino group of bound MgADP forms a hydrogen bond with the conserved Tyr-135 of the S1Dc (Fisher et al., 1995).

The importance of specific interactions of the base moiety with the protein to produce energy coupling is further underscored by the data derived from studies using non-nucleoside triphosphates. There appears to be a specific requirement of an ortho nitro group at the aryl ring of non-nucleoside (nitrophenylamino) class of triphosphates to support contraction (Wang et al., 1993), suggesting that, in this analogue class, the oxygens of the ortho nitro group act as hydrogen bond acceptors to form a necessary specific interaction from a protein donor. The present results for the nucleoside class, however, suggest that, while a specific interaction of the heterocyclic base with the protein is necessary to generate force, torsional movement involving the base may be an additional prerequisite for energy coupling as suggested by Takenaka et al. (1978). In this connection, it is of interest to examine the conformations of bound MgADP in the ternary complexes formed with *Dictyostelium discoideum* S1 motor domain and BeF_x or AlF_4^-. While the number of specific interactions between the protein and the bound MgADP is believed to remain essentially unchanged in these complexes, alterations in the structure of the bound nucleotide diphosphate can be clearly discerned (Table 2). These differences apply to the torsional angles of the *N*-glycosidic bond and the C5′−C4′ bond and can be more readily seen when these two S1 bound states of MgADP are compared with their adenine rings overlaid as shown in Figure 8. In this comparison, the differences between the β-bridging oxygen atoms and between the two β-phosphorus atoms are ∼1.9 and ∼1.45 Å, respectively. This comparison clearly demonstrates that the structure of the nucleotide does not remain static, but that it does change as it is processed at the active site of S1, and this involves changes in the torsional angle, κ, of the *N*-glycosidic bond.

In contrast to the properties of the S1 steady-state intermediates formed by the analogue triphosphate and MgATP (that is, common structures but different stabilities), the available evidence for their corresponding S1 Mg^{2+} diphosphate complexes points to their having different structures but similar stabilities. For example, the S1·Mg·-enf-ADP complex shows the ability neither to induce an enhancement in the S1 tryptophyl fluorescence nor to expose the SH2 thiol to react with added NEM (Figure 3), both of which are benchmark properties observed with the S1·-MgADP complex (Werber et al., 1972; Yamaguchi & Sekine, 1966). Therefore, the structures of the binary S1 complexes and these diphosphates differ. On the other hand, the binding affinity of Mg·enf-ADP for S1 appears to be as strong as that of MgADP, and, despite its inability to affect the SH2 reactivity, its binding does cause an increase in the reactivity of the SH1 thiol, indicating that the SH1, SH2 helix region is perturbed by its binding to the active site. Since their interaction with S1 most likely occurs through the Mg^{2+} diphosphate moiety, it is not surprising that the stabilities of these binary complexes are not dissimilar. The differences in their S1 binary complexes, therefore, are likely to reflect changes related to the remainder of the nucleotide structure, and, as the adenine ring is common, these structural difference must arise by some influence imposed by the enofuranosyl ring substitution. Since, as pointed out earlier, this substitution would not bring about a large change in the steric relationships between the diphosphate and adenine ring, it appears that these differences in the S1·MgADP and S1·

Mg•enf-ADP complexes most likely arise due to the freer rotational freedom associated with the N9−C1′ bond of the latter.

The structural differences in the S1 binary complexes formed with these Mg^{2+} nucleoside diphosphates also appear to have a dramatic influence on the rate of ternary complex formation with various P_i analogues. Since there appears to be a strong correlation in an analogue triphosphate's ability to support actomyosin contraction (Regnier et al., 1993; Pate et al., 1993) or motility (Homsher et al., 1993) and the ability of its diphosphate to be trapped in S1 by various P_i analogues (Bobkov & Levitsky, 1995), it was anticipated that Mg•enf-ADP would be readily trapped in S1 by this procedure. However, the data show that, with V_i as the P_i analogue, the rate of ternary complex formation is two orders of magnitude slower for enf-ADP as opposed to ADP (slower rates were also observed with other P_i analogues but the differences were smaller in these cases). Since the chemical modification data indicate that the affinity of Mg•enf-ADP for S1 is at least as high as that for MgADP, the reduced formation rate for ternary complex formation cannot be attributed to low amounts of the binary complex present when Mg•enf-ADP is present. It appears, then, that it is the binding of the P_i analogue to the binary complex which is rate limiting in the case of the S1•Mg•enf-ADP complex, since the amount of ternary complex formed does vary significantly with the concentration of the P_i analogue used (Figures 6 and 7). This lower binding most likely is related to the observed structural difference in the S1•Mg^{2+} nucleoside diphosphate complexes, indicated by the fluorescence and chemical modification data, and may be related to the faster release of P_i observed for the steady-state S1 intermediate formed with Mg•enf-ATP and to the lower overall stability of the S1 ternary complexes formed with Mg•enf-ADP and the various P_i analogues.

The DSC data indicate that both V_i and BeF_x are capable of stabilizing the S1 structure to thermal denaturation in the presence of Mg•enf-ADP since they both show a new thermal transition with higher T_m's then observed for the binary complexes. Thus, despite the lower formation rates for these ternary complexes, once formed they show a thermal stability comparable to those formed with MgADP and the corresponding P_i analogues. As expected from the higher rate of decay of the steady-state intermediate formed during the hydrolysis of Mg•enf-ATP by S1, these ternary complexes stabilized by the P_i analogues show lower stability at 4 °C than those containing trapped MgADP, suggesting that their coordination to the S1 bound Mg•enf-ADP is weaker at this temperature. This lower stability cannot be attributed to the loss of the hydroxyl groups from the 2′ and 3′ carbons since the corresponding 2′-deoxy-, 3′-deoxy and 2′,3′-dideoxyadenosine diphosphates are well trapped in S1 by either V_i or BeF_x, and they show comparable stabilities at 4 °C to that of the corresponding complexes formed with MgADP (Pavlov & Levitsky, unpublished data). Therefore, it may be concluded that the lower stabilities of the corresponding complexes formed with Mg•enf-ADP arise from either the conformational effect of the planar enofuranosyl ring and/or from the enhanced torsional freedom at the N9−C1′ bond.

REFERENCES

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

Bobkov, A. A., & Levitsky, D. I. (1995) *Biochemistry 34,* 9708−9713.

Bobkov, A. A., Khovorov, N. V., Golitsina, N. L., & Levitsky, D. I. (1993) *FEBS Lett. 332,* 64−66.

Cooke, R. (1986) *CRC Crit. Rev. Biochem. 21,* 53−118.

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

Godfrey, J. E., & Harrington, W. F. (1970) *Biochemistry 9,* 886−893.

Goldman, Y. E. (1987) *Annu. Rev. Physiol. 49,* 637−654.

Goody, R. S., & Isakov, M. (1986) *Tetrahedron Lett. 27,* 3599−3602.

Greene, L. E., & Eisenberg, E. (1980) *J. Biol. Chem. 255,* 543−548.

Haley, B., & Yount, R. G. (1972) *Biochemistry 11,* 2863−2871.

Harrington, W. F., Reisler, E., & Burke, M. (1975) *J. Supramol. Struct. 3,* 112−124.

Homsher, E., & Millar, N. C. (1990) *Annu. Rev. Physiol. 52,* 875−896.

Homsher, E., Regnier, M., & Tejeda, S. (1993) *Biophys. J. 64,* A250.

Hutcheon, W. L. B., & James, M. N. G. (1974) *Acta Crystallogr. B30,* 1777−1782.

Kielley, W. W., & Bradley, L. B. (1956) *J. Biol. Chem. 218,* 653−659.

Kovacs, T., & Otvos, L. (1988) *Tetrahedron Lett. 29,* 4525−4528.

Krawczyk, S. H., & Townsend, L. B. (1989) *Nucleosides Nucleotides 8,* 97−115.

Lai, T. F., & Marsh, R. E. (1972) *Acta Crystallogr. B28,* 1982−1989.

Levitsky, D. I., Shnyrov, V. L., Khvorov, N. V., Bukatina, A. E., Vedenkina, N. S., Permyakov, E. A., Nikolaeva, O. P., & Levitsky, D. I. (1992) *Eur. J. Biochem. 209,* 829−835.

Lymn, R. W., & Taylor, E. W. (1971) *Biochemistry 10,* 4617−4624.

Morita, F., & Yagi, K. (1966) *Biochem. Biophys. Res. Commun. 22,* 297.

Nakamaye, K. L., Wells, J. A., Bridenbaugh, R. L., Okamoto, Y., & Yount, R. G. (1985) *Biochemistry 24,* 5226−5235.

Pate, E., Franks-Skiba, K., White, H., & Cooke, R. (1993) *J. Biol. Chem. 268,* 10046−10053.

Regnier, M., Bostani, P., & Homsher, E. (1993) *Biophys. J. 64,* A250.

Reisler, E., Burke, M., & Harrington, W. F. (1974) *Biochemistry 13,* 2014−2022.

Rosenfeld, S. S., & Taylor, E. W. (1984) *J. Biol. Chem. 259,* 11908−11919.

Sekiya, K., & Tonomura, Y. (1967) *J. Biochem. (Tokyo) 61,* 787−795.

Shimizu, T., Furusawa, K., Ohashi, S., Toyoshima, Y. Y., Okuno, M., Malik, F., & Vale, R. D. (1991) *J. Cell Biol. 112,* 1189−1197.

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

Takenaka, H., Ikehara, M., & Tonomura, Y. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75,* 4229−4233.

Taylor, E. W. (1979) *CRC Crit. Rev. Biochem. 7,* 103−164.

Tonomura, Y. (1973) *Muscle Proteins, Muscle Contraction and Cation Transport,* pp 259−271, University Park Press, New York.

Toyoshima, Y. Y., Kron, S. J., McNally, E. M., Niebling, K. R., Toyoshima, K., & Spudich, J. A. (1987) *Nature 328,* 536−539.

Wang, D., Pate, E., Cooke, R., & Yount, R. G. (1993) *J. Muscle Res. Cell Motil. 14,* 484−497.

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

Werber, M. M., Szent-Gyorgyi, A. G., & Fasman, G. D. (1972) *Biochemistry 11,* 2872−2883.

White, H. D. (1982) *Methods Enzymol. 85B,* 698−708.

Yamaguchi, M., & Sekine, T. (1966) *J. Biochem. (Tokyo) 59,* 24−33.

Yount, R. G., Babcock, D., Ballantyne, W., & Ojala, D. (1971) *Biochemistry 10,* 2484−2489.

BI960597O