Structural Basis for Actomyosin Chemomechanical Transduction by Non-Nucleoside Triphosphate Analogues†

Damodaragounder Gopal,‡ Andrey A. Bobkov,§,|| James P. Schwonek,⊥ Charles R. Sanders,⊥ Mitsuo Ikebe,⊥ Dmitrii I. Levitsky,§ 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 117071, Russia, and Department of Physiology and Biophysics, School of Medicine, Case Western Reserve University, Cleveland, Ohio 44106

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ABSTRACT: Methylation of 2-[(2,4-dinitrophenyl)amino]ethyl triphosphate (dNOTP) was found to abolish its ability to support actin sliding in the *in vitro* motility assay. A comparative study of the interaction of myosin subfragment 1 (S1) and actoS1 with methylated (MdNOTP) and non-methylated dNOTP was undertaken. Both analogues were shown to be substrates for S1 NTPase in the presence of K^+/EDTA, Ca^{2+}, or Mg^{2+}, although their rates of hydolysis in the presence of the divalent cations were significantly greater than that occurring for ATP. However, actin had only a marginal effect on the rate of hydrolysis of MdNOTP, in sharp contrast to its effect on the hydrolysis of dNOTP and ATP which were quite similar. Moreover, while dNODP is able to form stable ternary S1 complexes with orthovanadate (V_i) or berylium fluoride (BeF_x), whose formation results in increased thermal stability of S1, the methylated diphosphate analogue was unable to do so. These differences can be related to methylation-induced changes in the conformation of dNOTP indicated by molecular-modeling approaches. These studies suggest that methylation prevents the specific interaction of the aryl ring of dNOTP with S1 in the adenine binding region necessary for the formation of the force-producing intermediate (M. D. P*) during the S1 Mg^{2+}-NTPase cycle.

It is well established that muscle contraction occurs as a consequence of the hydrolysis of MgATP by myosin in the presence of actin (Huxley, 1967; Huxley & Simmons, 1971). The biochemical cycle of this interaction has been well-studied in solution with myosin S1[1] and actin (Lymn & Taylor, 1971; Bagshaw & Trentham, 1974; Johnson & Taylor, 1978) and in skinned fibers using caged ATP [reviewed in Homsher and Millar (1990)], and there is a high degree of correspondence of many of the pre-steady state rate constants in solution and in the fiber. An intriguing aspect of chemomechanical transduction in the actomyosin motor system is the structural requirements of the substrate molecule that allow the free energy associated with the hydrolysis of its terminal phosphoryl group by S1 to be coupled by actomyosin to produce force or movement. Tonomura and his associates studied this aspect of the problem in detail by examining the naturally occurring nucleoside triphosphates and by making systematic substitutions at each of the three chemical moieties present: the heterocyclic base, the ribose ring, and the triphosphate chain [reviewed in Tonomura (1973)]. The observation that the non-nucleotide analogue NANTP, synthesized by Nakamaye et al. (1985), could support tension generation in skinned fibers (Pate et al., 1991) has led to renewed interest in the nature of the structure of these synthetic non-nucleotide analogues that enable them to support energy transduction. These non-nucleotide analogues are characterized by not having a ribose ring and not having a natural heterocyclic base, and in their place is an ethyl linker and an aryl ring.

In an examination of the effects of changes in the ring or linker region on the ability of a NANTP-like analogue to support contraction, it was found that the essential requirements appeared to be a nitro group at the 2-position of the ring (ortho) and an ethyl linker (Wang et al., 1993). However, these requirements were based on a rather limited number of substitutions at the 2-position and on the effects of increasing the ethyl linker by one methylene group to a propyl with both substitutions abolishing chemomechanical transduction. In the present paper, we have sought to obtain more information about these structural requirements. To do this, we have synthesized an analogue in which the two essential components, the 2-nitro and the ethyl linker, have been retained and the only change has been made at the nitrogen of the aryl amino group linking the aryl ring to the ethyl linker of the analogue. In this case, the substitution is a methyl substitution for the amino proton.

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* Author to whom correspondence should be addressed.

‡ Department of Biology, Case Western Reserve University.

§ Russian Academy of Sciences.

|| Present address: Molecular Biology Institute, University of California, Los Angeles, CA 90024.

⊥ Department of Physiology and Biophysics, Case Western Reserve University.

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[1] Abbreviations: S1, myosin subfragment 1; V_i, orthovanadate; BeF_x, berylium fluoride; dNOTP, 2-[(2,4-dinitrophenyl)amino]ethyl triphosphate; MdNOTP, 2-[methyl(2,4-dinitrophenyl)amino]ethyl triphosphate; dNODP and MdNODP are the diphosphate forms of dNOTP and MdNOTP, respectively; DSC, differential-scanning calorimetry; Hepes, *N*-(hydroxyethyl)piperazine-N′-2-ethanesulfonic acid; NANTP, 2-[(4-azido-2-nitrophenyl)amino]ethyl triphosphate; NTP and NDP refer to triphosphate and diphosphate of naturally occurring nucleosides and analogues; Tris, tris(hydroxymethyl)aminomethane.

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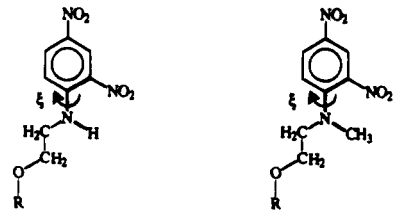


1a: R = H
1b: R = Monophosphate (dNOMP)
1c: R = Diphosphate (dNODP)
1d: R = Triphosphate (dNOTP)

2a: R = H
2b: R = Monophosphate (MdNOMP)
2c: R = Diphosphate (MdNODP)
2d: R = Triphosphate (MdNOTP)

FIGURE 1: Chemical structures of precursor alcohols (**1a** and **2a**), monophosphates (**1b** and **2b**), diphosphates (**1c** and **2c**), and triphosphates (**1d** and **2d**) of synthetic non-nucleotide unmethylated and methylated analogues. **1d**, named dNOTP in this paper, corresponds to DNPhAETP in the work of Wang et al. (1993).

Our results show that this analogue can still act as a substrate for S1 and actoS1 MgNTPase, but its steady state NTPases are markedly modified in comparison to that of its unmethylated form. Moreover, it is unable to support actin sliding in the *in vitro* motility assay using adsorbed myosin. To obtain insights as to the nature of this altered behavior and lack of contractile functionality, we have also examined the nature of the interaction of its diphosphate form with S1 in the presence and absence of V_i and BeF_x anions. We did this by measuring the abilities of these anions to trap the analogue and to generate inhibitory complexes with S1. Additionally, differential-scanning calorimetry was used to monitor the ability of these analogues together with V_i and BeF_x to generate the structural change in S1 indicative of increased thermal stability of the protein. Molecular modeling using energy minimization methods suggests that the methylated and non-methylated forms of this analogue are likely to have markedly different conformations, with the presence of the methyl group restricting the orientation of the aryl ring at a configuration rotated about 90° from that observed for the unmethylated analogue.

MATERIALS AND METHODS

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). Actin was prepared from the rabbit skeletal muscle as described by Spudich and Watts (1971).

Synthesis of Analogues. 2-[(2,4-Dinitrophenyl)amino]-ethanol (**1a**, Figure1) and its *N*-methyl analogue **2a** (Figure 1) were synthesized by the reaction of 2,4-dinitrofluorobenzene with an excess of aminoethanol and (*N*-methylamino)-ethanol, respectively. The syntheses of triphosphates (**1d** and **2d**) and diphosphates (**1c** and **2c**) were carried out using the procedure of Yoshikawa et al. (1967) and Hoard and Ott (1965).

Preparation of S1·NDP·V_i and S1·NDP·BeF_x Complexes. This was done in a manner similar to that reported by Goodno (1982) and Werber et al. (1992). In order to obtain the complexes of S1 with V_i or BeF_x and ADP or other diphosphates, S1 (1 mg/mL) was incubated with 1 mM $MgCl_2$, 0.2 mM V_i, or 0.2 mM BeF_x (5 mM NaF + 0.2 mM $BeCl_2$) and 0.2 mM ADP or other diphosphates for 30 min at 20 °C in a medium containing 30 mM Hepes, pH 7.3.

Trapping and Stability of the S1·NDP·BeF_x Complex. Trapping of NDP's was done by incubation of S1 for 45 min at 25 °C in 0.01 M Tris, 0.04 M KCl, 1 mM NDP, 3 mM $MgCl_2$, 5 mM NaF, and $BeCl_2$ (0.7 mM). The protein was then kept at 4 °C for 30 min. The samples were made 1.0 mM with respect to ATP 10 min prior to removal of excess reagents. Excess reagents were removed by two gel filtration centrifugations at 5 °C through Sephadex G-50 equilibrated with 0.01 M Tris, and 0.25 M Na_2SO_4 at pH 7.0 and then placed in a water bath at 20 °C. The protein was examined at different times of incubation by passage through a Beckman ultraspherogel SEC 4000 column at 1 mL/min flow rate to analyze the release of NDP from S1 by the monitoring of the eluent at 360 nm (dNODP) and 388 nm (MdNODP).

ATPase and NTPase Activities. The K^+/EDTA-NTPase activity of the S1 was determined from the release of P_i (Panusz et al., 1970) at 25 °C in medium containing 0.04 mg/mL S1, 1 mM ATP, 0.5 M KCl, 5 mM EDTA, and 50 mM Tris-HCl (pH 7.5). The reaction was initiated by addition of NTP and stopped after 5−10 min of incubation by addition of $HClO_4$.

The actin-activated MgNTPase was assayed in 4 mM KCl, 50 mM imidazole, 3 mM $MgCl_2$, and 3 mM triphosphates, pH 7.0, at 25 °C at a myosin S1 concentration of 0.05 mg/mL. The amount of phosphate release was measured spectrophotometrically by the method of White (1982). The S1 NTPase assays were done at 25 °C by the method of Nakamaye et al. (1985).

In Vitro Motility Assays. 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 or recorded on videotape. Speeds of actin displacement were analyzed by replay of the video tapes.

Differential-Scanning Calorimetry (DSC). Calorimetric measurements were carried out on a differential adiabatic scanning microcalorimeter DASM-4 (Russia) as described earlier (Levitsky et al., 1992). The heating rate was 1 K/min. The denaturation enthalphy, ΔH_{cal}, was calculated from the area under the heat sorption curve.

Molecular Modeling. Molecular mechanics of the dinitrophenyl monophosphate analogues, dNOMP and MdNOMP (Figure 1), were carried out on a Silicon Graphics (Mountain City, CA) IRIS 4D-TG33 instrument using routines provided by BIOGRAF (Molecular Simulations, Waltham, MA). The Dreiding II force field (Mayo et al., 1990) was used with modifications to the H bond potential; amine and resonant carbon donating H bond maximum energies were set at 3.0 and 1.5 kcal/mol, respectively.

RESULTS

(1). In Vitro Motility Assay and Hydrolysis of dNOTP and MdNOTP by S1 and actoS1 NTPases. The sliding velocities of actin over myosin adsorbed on nitrocellulose-coated glass cover slips in the presence of MgATP and dNOTP were 4.5 (±0.86) and 2.1 (±0.32) μm/s, respectively, and 0 with MdNOTP. The lack of motility observed with the latter analogue suggests either that the analogue is not a substrate for S1 and actoS1 NTPases or that it is unable to produce a force-generating intermediate complex (S1**·MgNDP·Pi). To investigate these possibilities, the hydrolysis of MdNOTP by S1 in the absence and in the presence of actin was next examined in comparison with ATP and dNOTP, and the results are shown in Tables 1 and 2 and in

Table 1: Hydrolysis of Analogues by S1 (μmol of P_i min^{-1} mg^{-1})

assay system	ATPase	dNOTPase	MdNOTPase
NH_4^+ EDTA	14.2 ± 0.03	4.3 ± 0.09	1.1 ± 0.02
Ca^{2+}	1.56 ± 0.03	2.6 ± 0.05	3.38 ± 0.10
Mg^{2+}	0.027 ± 0.001	0.12 ± 0.002	0.53 ± 0.01

Table 2: Kinetic Properties of Hydrolysis of ATP and NTP Analogues by S1 in the Presence and Absence of Actin

	rate (μmol of P_i min^{-1} mg^{-1})			
analogue	without actin	with actin	actin activation	K_{app} (μM)
ATP	0.027 ± 0.001	8.4 ± 0.60	311	114 ± 31
dNOTP	0.12 ± 0.001	9.4 ± 4.6	78	151 ± 78
MdNOTP	0.53± 0.005	1.71 ± 0.34	3.2	4.1 ± 1.5

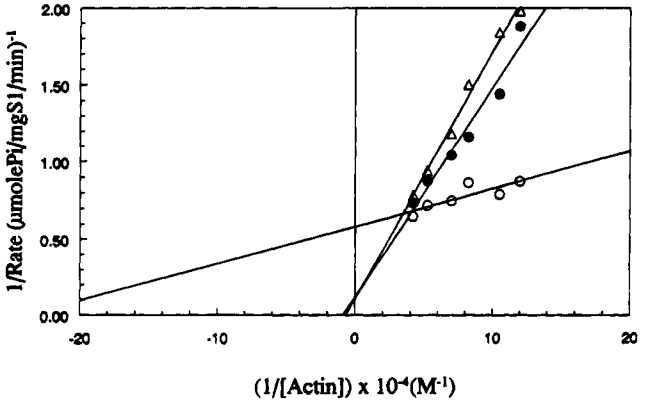


FIGURE 2: Double reciprocal plots of hydrolysis rates for S1 NTPases versus F-actin concentration. (●) ATP, (△) dNOTP, and (○) MdNOTP. Assays were performed as described in Materials and Methods.

Figure 2. The intrinsic rates of hydrolysis of dNOTP and MdNOTP alone or in the presence of F-actin only were ~1.0 and 0.7 nmol/min, respectively, which are negligible compared to the rates observed in the presence of S1 with or without actin. Furthermore, no significant change was observed for the turnover rate for the S1 Mg^{2+}MdNOTPase over a concentration range of 1 to 4 mM for this substrate, indicating that S1 is saturated at the former concentration. Therefore, it is evident that S1 alone is capable of hydrolyzing MdNOTP in the presence and absence of divalent cations. Both analogues show higher rates of hydrolysis by S1 in the presence of Ca^{2+} and Mg^{2+} ion and lower hydrolysis rates in the absence of divalent cations compared to ATP (Table 1). These effects are more pronounced in the case of the methylated analogue.

The effect of actin on the hydrolysis rates of these analogues by S1 (shown in Table 2 and Figure 2) indicates that actin has only a marginal effect on the rate of hydrolysis of MdNOTP, in sharp contrast to its effect on the hydrolysis of dNOTP and ATP which are quite similar. These data indicate that, under steady state conditions, S1 binds more strongly to actin in the presence of the methylated analogue than in the presence of dNOTP and ATP. Turbidometric analyses of the actoS1 interaction showed that Mg^{2+} MdNOTP at 1 mM levels induced an initial rapid full dissociation of the complex, indicating that the binding of this analogue does produce dissociation of this protein complex (data not shown). It is, therefore, possible to conclude that the methylated analogue can bind and be hydrolyzed by S1 in the presence and absence of actin but that its binding to actin under steady state conditions occurs with much higher affinity than observed with ATP and dNOTP. These results point to an altered interaction of this analogue with S1 and actoS1 precluding its ability to form significant concentrations of S1 molecules weakly binding to actin. This altered interaction most likely corresponds to the inability of MdNOTP to form a force-generating intermediate during steady state conditions analogous to the S1**·MgADP·Pi state.

Table 3: Relative ATPase Activities of S1 in the Presence of Different Diphosphates (0.2 mM) and V_i (0.2 mM) or BeF_x (0.2 mM)

	K^+ EDTA-ATPase activity (%)
S1	100
S1 + ADP	96.0
S1 + dNODP	87.5
S1 + MdNODP	79.5
S1 + ADP + V_i	7.0
S1 + dNODP + V_i	9.0
S1 + MdNODP + V_i	69.5
S1 + ADP + BeF_x	10.5
S1 + dNODP + BeF_x	19.5
S1 + MdNODP + BeF_x	73.5

[a] 100% K^+-EDTA-ATPase activity refers to 3.6 μmol of P_i mg^{-1} min^{-1}.

(2). MdNODP and dNODP Interactions with S1 in the Presence of V_i and BeF_x. The previous data (Table 2 and Figure 2) suggest that MdNOTP, unlike ATP and dNOTP, is unable to form significant amounts of the weakly actin binding state corresponding to the S1**·MgADP·Pi intermediate under steady state conditions. Since it is well-established that stable S1 complexes with MgADP and Pi analogues such as V_i, BeF_x, and AlF_4^- can be used as good models for this transition state (Goodno, 1979, 1982; Werber et al., 1992; Phan & Reisler, 1992; Henry et al., 1993; Phan et al., 1993) and lead to marked inactivation of the ATPase of the protein, we examined the ability of MdNODP and dNODP to form these complexes. The data presented in Table 3 give the extent of inactivation observed for S1 after incubation of these analogues in the presence of V_i and BeF_x. In the absence of V_i or BeF_x, the binding of ADP and the analogue diphosphates to S1 inhibited only slightly the S1 ATPase. On the other hand, the marked inhibition observed in the presence of V_i and BeF_x for ADP and dNODP points to the formation of stable inhibitory complexes. The failure to observe similar high levels of inhibition with MdNODP in the presence of V_i and BeF_x suggests that this diphosphate is poorly, if at all, trapped in S1 by these Pi analogues.

The inability of the Pi analogues to trap MdNODP in S1 could conceivably arise from the fact that this diphosphate either does not bind to S1 or binds with very low affinity. While the DSC data to be presented below indicate that MdNODP in the presence of Mg^{2+} does bind to S1, we have estimated the binding by measuring the MdNODP concentration dependence on the rate of inactivation of the S1 K^+/EDTA ATPase activity by reaction with *N*-ethylmaleimide. The dissociation constant for the binding of Mg^{2+} MdNODP to S1 was estimated to be about 2.25×10^{-4} M by this analysis (data not shown). While this is significantly lower than that for binding of MgADP to S1 [10^{-5}–10^{-6} M at 22 °C; Greene and Eisenberg (1980) and Webb and Trentham (1983), respectively], under conditions of the trapping studies,

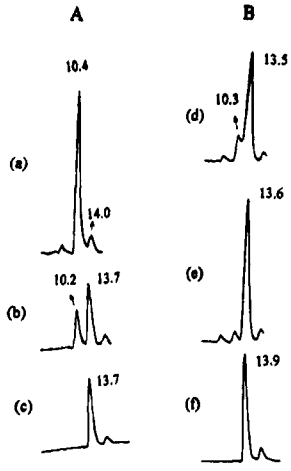


FIGURE 3: Examination of the stability of complexes formed in S1 with NDP analogues in the presence of BeF_x. The complexes were isolated by gel filtration centrifugation at 5 °C and then placed in a water bath at 20 °C, and after a 5 min incubation, samples were then removed at the indicated times for analyses by high-performance size exclusion chromatography as described in Materials and Methods. (A) S1·dNODP·BeF_x: (a) 5 min of incubation, (b) 120 min of incubation. (B) S1·MdNODP·BeF_x: (d) 5 min of incubation, (e) 25 min of incubation. (c) and (f) correspond to the analogues in the absence of S1. The elution times for S1 and the free analogues are 10.3 (±0.1) and 13.7 (±0.2) min, respectively, as indicated in the figure.

approximately 80% of the S1 should be occupied by MdNODP.

These observations do not imply that MdNODP cannot form a ternary complex with S1 in the presence of the Pi analogues but, instead, do imply that this ternary complex has very low stability. Further support for this suggestion is provided in the results of Figure 3. These data show that MdNODP is released from S1 in the presence of BeF_x much faster than dNODP. Since there is still residual MdNODP associated with the protein, even after addition of ATP to 1 mM and despite two centrifugations through Sephadex G-50, the binding of this diphosphate is enhanced in the presence of BeF_x, but the overal stability of this ternary complex is much poorer than those formed with ADP and dNODP. Therefore, the combined data suggest that MdNODP is unable to form a stable complex with S1 in the presence of BeF_x and V_i.

(3). DSC Studies of S1 Complexes with dNODP and MdNODP. It has been shown previously that differential-scanning calorimetry (DSC) is a sensitive probe for structural changes induced in S1 by binding of nucleoside diphosphates and by formation of their S1·NDP stabilized complexes with V_i or BeF_x (Levitsky et al., 1992; Bobkov et al., 1993; Bobkov & Levitsky, 1995). Reversible interactions with nucleoside diphosphates generally lead to an increase in the cooperativity of the thermal transition, whereas the formation of the stable ternary complexes is expressed by significant increases in the thermal stability of S1.

Figure 4 shows data on the thermally induced unfolding of S1 and of the complexes of S1 with dNODP in the absence and in the presence of V_i. The binding of dNODP alone to S1 has practically no influence on the temperature of the thermal transition (48.7 °C) but increases its sharpness (cooperativity). This effect of dNODP is very similar to that found earlier for the binding of ADP and other nucleoside diphosphates to S1 (Levitsky et al., 1992; Bobkov &

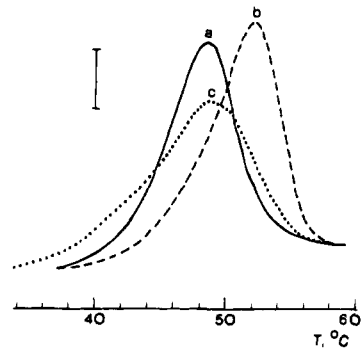


FIGURE 4: Temperature dependence of excess heat capacity (ΔC_p) for S1 (a) in the presence of 0.2 mM dNODP and (b) in the presence of 0.2 mM dNODP and 0.2 mM V_i. (c) is the control S1 without additions. S1 concentration was 1 mg/mL. Conditions: 30 mM Hepes, pH 7.3; 1 mM $MgCl_2$. Vertical bar corresponds to 50 kJ K^{-1} mol^{-1}.

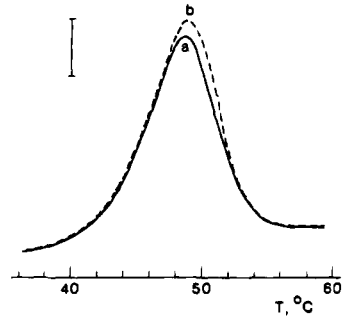


FIGURE 5: Temperature dependence of excess heat capacity (ΔC_p) for S1 (a) in the presence of 0.2 mM MdNODP and (b) in the presence of 0.2 mM MdNODP and 0.2 mM V_i. Conditions were as in Figure 4. Vertical bar corresponds to 50 kJ K^{-1} mol^{-1}.

Levitsky, 1995), and this can therefore be used to monitor analogue binding to the protein. Formation of the S1·dNODP·V_i complex, on the other hand, increases the temperature of the thermal transition to 52.2 °C (Figure 4). Thus, the DSC scans can clearly distinguish the effects caused by the binding of dNODP alone to S1 from those involving formation of the S1·dNODP·V_i complex. Essentially, the same result (a significant shift of the thermal transition) was obtained on the complex of S1 with dNODP in the presence of BeF_x (data are not shown). Thus, formation of these stable ternary complexes is accompanied by conformational changes in S1 which are reflected in the pronounced shift of the thermal transition.

The data in Figure 5 show that MdNODP can bind to S1 in a manner similar to that of dNODP as reflected in the increased cooperativity of the S1 thermal transition. However, in contrast to dNODP, this transition is quite insensitive to both V_i and BeF_x and is essentially the same as that observed for S1 with MdNODP alone (Figure 5). These data indicate that neither V_i nor BeF_x can induce any significant additional conformational changes in the S1·MdNODP complex, suggesting that this analogue is unable to form a stable complex with S1 and V_i or BeF_x of the type observed with dNODP.

The main thermodynamic parameters of the thermal denaturation (denaturation temperature, T_d, and denaturation enthalpy, ΔH_{cal}) obtained from the DSC measurements on various complexes of S1 with compounds studied are summarized in Table 4. As mentioned above, T_d is the main parameter reflecting S1 conformational changes associated

Table 4: Thermodynamic Parameters Obtained from the DSC Data (Figures 4 and 5) for S1 and Its Complexes with dNODP and MdNODP in the Presence or in the Absence of V_i or BeF_x

	T (°C)	ΔH (kJ/mol)
S1	48.7 ± 0.1	1341 ± 67
S1 + dNODP	48.7 ± 0.1	1313 ± 66
S1 + MdNODP	48.6 ± 0.1	1317 ± 66
S1 + dNODP + V_i	52.2 ± 0.1	1420 ± 71
S1 + MdNODP + V_i	49.0 ± 0.1	1426 ± 71
S1 + dNODP + BeF_x	52.0 ± 0.1	1386 ± 69
S1 + MdNODP + BeF_x	48.8 ± 0.1	1352 ± 69

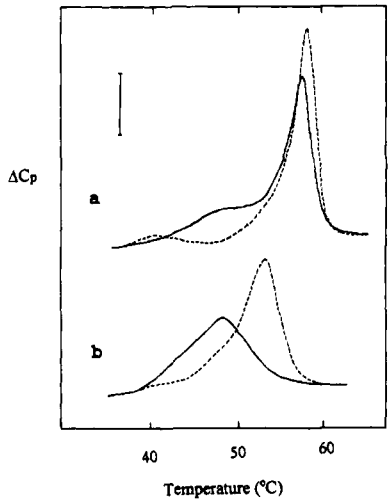


FIGURE 6: Temperature dependence of excess heat capacity for S1 in the complexes (a) S1·ADP·V_i and (b) S1·dNODP·V_i before and after removal of excess reagents. Curves shown by dotted lines were obtained for S1 in the presence of 0.2 mM ADP or dNODP and 0.2 mM V_i. Curves shown by unbroken lines were obtained after removal of excess ADP or dNODP and V_i from the complexes by dialysis against 30 mM Hepes, pH 7.3, containing 1 mM $MgCl_2$, at 4 °C for 24 h. Conditions were as in Figure 4. Vertical bar corresponds to 100 kJ K^{-1} mol^{-1}.

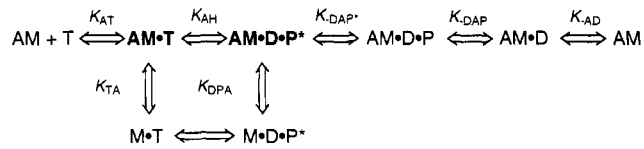
with the formation of stable complexes of S1 with nucleoside diphosphates and V_i or BeF_x. An analysis of T_d values shows that the effect of MdNODP is too negligible in comparison with the effect of dNODP.

The DSC method can also be used to examine the relative stability of the S1 complexes with nucleoside diphosphates and V_i or BeF_x (Bobkov & Levitsky, 1995), provided the calorimetric peaks associated with these complexes are distinguishable from those corresponding to nucleotide-free S1. The complexes with dNODP meet these criteria, and as shown in Figure 6, the complex S1·dNODP·V_i was completely dissociated after dialysis since only the thermal transition characteristic for the nucleotide-free S1 was then observed (Figure 6b). On the other hand, this effect was negligible for the S1·ADP·V_i complex (Figure 6a), indicating a much higher stability than that for the S1·dNODP·V_i complex. Similar results were observed for the S1·dNODP·BeF_x complex (data not shown).

DISCUSSION

The purpose of this study was to obtain insights into the nature of the structural requirements that enable the NANTP class of analogue to support actomyosin contractility. The effect of methylation at the aryl amino group was undertaken to examine a small perturbation of the analogue while two benchmark features thought to be essential for the ability of these analogues to support contraction were left unchanged: the requirement of the 2-nitro group and an ethyl linker. The data indicate that this relatively simple substitution has produced a dramatic effect on the chemomechanical competency of the analogue since it is unable to generate actin sliding over immobilized myosin in the standard *in vitro* motility assay. This failure to observe movement could arise from the inability of this analogue to bind to and, therefore, to act as substrates for S1 or actoS1. However, the results shown in Table 1 indicate that MdNOTP is a reasonable substrate for these proteins since the hydrolysis of MdNOTP in the absence of these proteins or with F-actin alone is extremely low (~1.0 nmol/min) and cannot account for the higher rates observed in their presence. Furthermore, the ability of 1 mM concentrations of Mg^{2+} MdNOTP to dissociate actoS1 fully (data not shown) makes it unlikely that the failure of MdNOTP to support contraction can be attributed to the inability of the analogue to bind with reasonable affinity to S1 or to actoS1.

The normal biochemical cycle occurring with actomyosin MgATPase is illustrated below using the following mechanism (and nomenclature) of White et al. (1993):



with the isomerization step (**AM·D·P*** to AM·D·P) being responsible for force generation.

Differences in an analogue's ability to support contraction must signify changes in some of the rate and equilibrium constants of this basic mechanism, and these changes in turn must reflect alterations in the interactions of these analogues with the protein in certain steps of the cycle. While the rate steps affected in the S1 and actoS1 NTPases remain to be determined by pre-steady state kinetic analyses, it is clear that the methylated analogue in the presence of Mg^{2+} has a higher turnover rate than that of the unmethylated analogue and of MgATP (Table 1) in the absence of actin. This indicates either that the rate of the transition from the M·D·P* to the next state is still rate-limiting but accelerated for MdNODP or that another step in the cycle is now rate-limiting. In either event, it is unlikely that, for the methylated analogue, the concentration of its M·D·P* intermediate would be sufficient to allow for high concentrations of the weakly actin-binding preforce intermediate AM·D·P* to occur. The poor actin activation of the S1 hydrolysis of MdNOTP can be attributed to a very low apparent K_{actin} (Figure 2 and Table 2), indicating that, in the presence of this analogue, the binding of actin is very tight and that weak binding complexes characteristic of actoS1 systems with MgATP are not present or present at very low concentrations under steady-state conditions. These combined results indicate that MdNOTP is unable to produce the long-lived M·D·P* intermediate during its hydrolysis by S1.

Since it is generally assumed that the ternary complexes formed between S1, ADP, and Pi analogues such as V_i and BeF_x are structurally related to the M·D·P* state (Goodno, 1979, 1982; Werber et al., 1992; Phan & Reisler, 1992; Henry et al., 1993; Phan et al., 1993), it would not be unexpected that contraction would be related to an analogue's

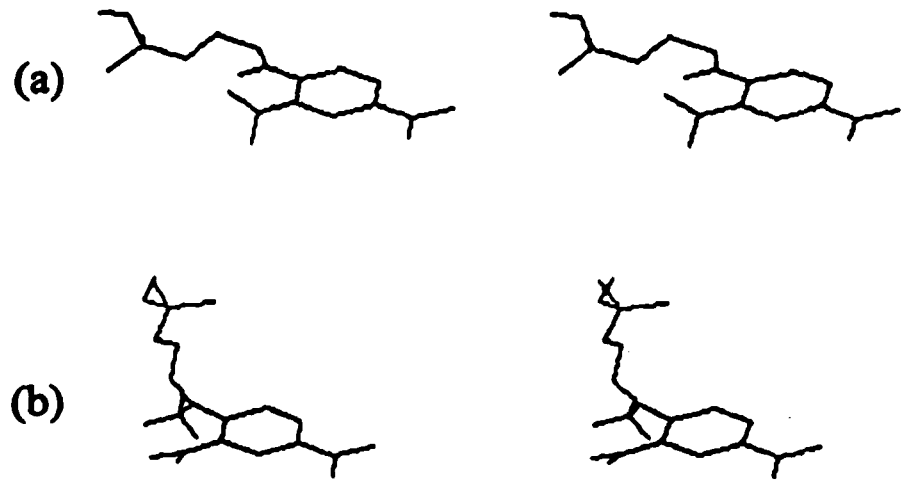


FIGURE 7: Stereoimages of energy minimized structures of (a) dNOMP and (b) MdNOMP.

capacity to stabilize the M·NDP·P* state and for its diphosphate to form stable ternary complexes with S1 and the common Pi analogues. The failure of MdNODP to stabilize the ternary S1 complex with the Pi analogues and its inability to support motility would be consistent with such a correlation. The lack of S1 ATPase inhibition by MdNODP in the presence of BeF_x and V_i (Table 3) is also consistent with the inability of this analogue to stabilize the ternary complex. This failure to inhibit cannot be attributed to the inability of MdNODP to bind to S1, since, even with the lower affinity (K_d equal to $\sim 2.3 \times 10^{-4}$ M), more than 80% of the S1 should have this diphosphate bound to it. The finding that some analogue coelutes with the protein (Figure 3), despite two gel filtration centrifugations, indicates that the binding of MdNODP to S1 is enhanced by the presence of BeF_x. This latter observation indicates that MdNODP does form a ternary complex with S1 in the presence of BeF_x, but its stability is considerably less than that formed with dNODP, as shown by its faster release from S1 upon removal of excess reagents.

The increased cooperativity of the S1 thermal transitions observed in the presence of dNODP and MdNODP (Figures 4 and 5) is also consistent with their binding to the protein. A similar effect has also been observed recently for the binding of ADP and other nucleoside diphosphates as well as PP_i to S1 (Bobkov & Levitsky, 1995). On the other hand, when V_i or BeF_x is present, a marked increase in the S1 denaturation temperature, T_d, was observed for dNODP but not for MdNODP (Table 4, Figures 4 and 5). The failure of MdNODP to increase S1 thermal stability is also consistent with the view that it cannot stabilize ternary complexes in the presence of these Pi analogues. A similar correspondence between MdNOTP and GTP exists with respect to their failure to support motility or contraction (Pate et al., 1993) and the inability of their corresponding diphosphates to stabilize S1 ternary complexes in the presence of V_i or BeF_x (Bobkov & Levitsky, 1995). These similarities suggest that neither analogue can generate the long-lived M·D·P* intermediate during their hydrolysis by S1, but this cannot be attributed to differences between the aryl ring of MdNOTP and the base moiety of GTP, since dNOTP, possessing the same aryl ring as MdNOTP, does support contraction (Wang et al., 1993) and motility (this work).

The main question which must be addressed is how the presence of the methyl group prevents chemomechanical transduction of this class of analogues. To obtain insights into this question, we have examined the effect of methylation on the conformation of dNODP by molecular modeling. Recent attempts to understand the structural basis that enables NANTP and CTP to support chemomechanical coupling have involved comparisons of these structures with that of ATP (Wang et al., 1993; Pate et al., 1993). In these cases, there appears to be a reasonable correlation to the structure of the natural substrate. On the basis of the chemical structures shown in Figure 1, a close correspondence between dNOTP and MdNOTP would be expected. The failure of MdNOTP to support motility, however, suggests an altered interaction with the protein arising from differences in the structures of dNOTP and MdNOTP incurred as a result of the methyl substitution of the amino hydrogen.

Energy minimization analyses for these two analogues in their monophosphate forms yielded values of 4 and 102°, respectively, for the dihedral angle ξ (Figure 1). These minimized energy conformations place the aryl rings in markedly different steric positions relative to the ethyl linker and phosphate group of these analogues as shown in Figure 7. X-ray diffraction analysis of the crystal form (dimer) of the alcohol (**1a**) yielded ξ of 3 and 6° (unpublished data), in good agreement with the energy minimization value. The low torsional angle of the unmethylated analogue is consistent with resonance delocalization of the amino nitrogen lone electron pair with the aromatic ring, and in this situation, the aryl amino proton is close enough to the oxygen of the ortho nitro group to form a H bond as has been postulated by Wang et al. (1993). In the absence of H bonding, rotation of the aryl ring about the C1′−N bond would be expected to have little if any energy barrier for NOMP. However, rotation about this bond for the methyl-substituted analogue from 102 to 4° was calculated to have a barrier significantly greater than 10 kcal/mol. Thus, this analysis indicates that the methyl substitution can lead to two distinct differences in the structure of MdNOTP with respect to dNOTP. Assuming that H bonding of the unmethylated analogue occurs, these two analogues should show quite distinct energy-minimized conformations in the unconstrained forms. On the other hand, in the absence of H bonding, there is a huge energy barrier for rotation about the dihedral angle in the case of the methylated analogue. Resolution of the contribution of these effects awaits studies with analogues in which torsional effects can be selectively restricted.

However, assuming that their triphosphate counterparts retain these differences upon interaction with the protein, it would not be unexpected that these substrates would be processed differently by the protein.

In conclusion, the present work has shown that dramatic changes in the chemomechanical efficiency of the NANTP class of analogues can result from a relatively small alteration in a region of the molecule not previously identified as being crucial for this property. This loss in efficiency appears to be associated with the inability of the methylated analogue to form the long-lived, force-generating M·D·P* intermediate during its hydrolysis by S1 and, in its diphosphate form, to produce stable complexes with V_i and BeF_x. These observations indicate that methylation has blocked an essential interaction with S1 needed to produce mechanical energy. The energy minimization data point to the importance of conformational and/or torsional contributions for the orientation of the aryl ring to interact specifically with the S1 adenine binding site to produce the force-generating intermediate.

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