Nucleoside diphosphate (NDP) kinase catalyzes the phosphorylation of ribo- and deoxyribonucleoside diphosphates into triphosphates. NDP kinase is also involved in malignant tumors and was shown to activate in vitro transcription of the c-myc oncogene by binding to its NHE sequence. The structure of the complex of NDP kinase with bound ADP shows that the nucleotide adopts a different conformation from that observed in other phosphokinases with an internal H bond between the 3'-OH and the β-OH made free by the phosphate transfer. We use intrinsic protein fluorescence to investigate the inhibitory and binding potential of nucleotide analogues phosphorylated in 3'-OH position of the ribose to both wild type and P64W mutant NDP kinase from Dictyostelium discoideum. Due to their 3'-phosphate, 5'-phosphoadenosine 3'-phosphate (PAP) and adenosine 3'-phosphate 5'-phosphosulfate (PAPS) can be regarded as structural analogues of enzyme-bound ADP. The KD of PAPS (10 μM) is three times lower than the KD of ADP. PAPS also acts as a competitive inhibitor toward natural substrates during catalysis, with a KI in agreement with binding data. The crystal structure of the binary complex between Dictyostelium NDP kinase and PAPS was solved at 2.8-Å resolution. It shows a new mode of nucleotide binding at the active site with the 3'-phosphate of PAPS located near the catalytic histidine, at the same position as the γ-phosphate in the transition state. The sulfate group is directed toward the protein surface. PAPS will be useful for the design of high affinity drugs targeted to NDP kinases.

Besides its role in intermediate metabolism of nucleotides, NDP kinase also plays a role in a variety of cellular functions. In Drosophila, the gene awd encoding NDP kinase is involved in development (4), and a point mutant has a conditional dominant phenotype known as Killer of prune (Kpn) (reviewed in Timmons and Shearn (5)). In humans, several NDP kinases encoded by the genes of the nm23 family play important roles in proliferation, tumor metastasis, and transcriptional regulation. Thus, nm23-H1 encoding NDP kinase A has been reported to be down regulated in some metastatic cell lines (6, 7), and transfection of nm23-H1 in melanoma cell lines could rescue the metastatic phenotype, indicating a possible role of nm23 as a metastasis suppressor gene (8). Immunohistochemical studies showed an increased expression level of NM23/NDP kinase in a variety of malignant tumors (46, 47), neuroblastoma (9), lymphoma (10, 11), and leukemia (12). The gene nm23-H2, encoding the B isoform of NDP kinase (12), was identified as a transcription factor for c-myc (13), a well known regulator of cell proliferation and differentiation (14). NDP kinase B was shown to bind oligonucleotides with sequences derived from the nuclelease hypersensitive element (NHE site, also termed PuF site) in the c-myc promoter (15). The implication of nm23-H2 in oncogenic proliferation is also indicated by the fact that nm23-H2 was found as one of the 20 genes expressed at the highest level among 300,000 transcripts analyzed in cells from human gastrointestinal tumors (16). Other nm23 genes were recently identified including DR-nm23, involved in apoptosis (17) and nm23-H4, which probably encodes a mitochondria NDP kinase (18).

The three dimensional structures of NDP kinases from several species have been determined at high resolution, (19–22) showing the conservation of both the subunit fold and of the active site. Eukaryotic NDP kinases are homohexamers made up of 17-kDa subunits with highly conserved sequences (23). The resolution of the structures of binary complexes between NDP kinase and ADP (24), dTDP (25), or GDP (26) demonstrated that the nucleotide binding site is different from other known nucleotide binding proteins. The enzyme-bound nucleotide adopts an unusual conformation due to the presence of an intramolecular hydrogen bond between the 3'-OH of the ribose and the oxygen of the β-phosphate (24). Thus the substitution of the 3'-OH by a phosphate group could lead to a high affinity inhibitor of NDP kinase.

So far, no tight binding inhibitor has been described for NDP kinases. Although cAMP was shown to inhibit NDP kinase activity (27, 28), its affinity for the enzyme was rather poor (0.5 mM). Flavonoid derivatives from plants were also reported as inhibitors of NDP kinase (29). The various functions of NDP kinase underline it as a potential therapeutic target. The discovery of efficient inhibitors could be the first step toward designing specific drugs targeted to NDP kinase. 3'-Phosphorylated nucleotides are potential inhibitors of NDP kinase and...
Inhibition of NDP Kinase by 3′-P Nucleotides

in particular adenosine 3′-phosphate 5′-phosphosulfate (PAPS), a metabolite involved in sulfonation of a variety of endogenous and exogenous chemicals (30). In this work, we used NDP kinase from the Dictyostelium discoideum that presents 62% sequence identity with the human enzyme (31) including a total conservation of the active site residues. Dictyostelium NDP kinase is best known at the structural level. It has a single tryptophan (Trp137), located at the proximity of the active site, and we previously showed that the quenching of the intrinsic fluorescence signal upon phosphorylation of the enzyme (32). We show here that PAPS inhibits NDP kinase activity with a high efficiency ($K_i = 30 \mu M$). The resolution of the structure of the complexes of NDPK with bound PAP and PAPS demonstrates a different mode of nucleotide binding to the active site.

**EXPERIMENTAL PROCEDURES**

**Materials—**ATP, dTDP, cAMP, lactate dehydrogenase, and pyruvate kinase were from Boehringer Mannheim. PAP and PAPS were from Sigma and (γ-32P)GTP from NEN Life Science Products. The mutation F64W in the Dictyostelium NDP kinase was made by site-directed mutagenesis as described in Schneider et al. (2).

**Enzyme Purification and Preparation of the Phosphorylated Enzyme—**Wild type and mutant Dictyostelium NDP kinases were overexpressed in Escherichia coli (XLI-Blue) using plasmid pmdkt as described previously (33) with small modifications. The cell extract was loaded at pH 8.4 onto a Q Sepharose matrix that retained only E. coli NDP kinase (34), and the flow-through was adsorbed on an Orange A matrix (Dyematrix, Amicon) at pH 7.5. After washing with Tris buffer, the enzyme was eluted by a gradient of NaCl (0 to 1.5 M) in 50 mM Tris-HCl, pH 7.5. After dialysis, the protein was concentrated with an Amicon ultrafiltration cell, equilibrated in 50 mM Tris-HCl, pH 7.5, and stored frozen at −20 °C. Protein concentration was determined using an absorbance coefficient of $\Delta A_{280nm} = 0.55$ for a 1 mg/ml solution. F64W mutant NDP kinase (2) was purified according to the same procedure. The absorption coefficient of the F64W mutant enzyme was $\Delta A_{280nm} = 0.85$ for a 1 mg/ml solution, according to the Gill equation for a folded protein in water (35). All proteins were purified to homogeneity as judged by SDS-polyacrylamide gel electrophoresis. Enzyme concentration was expressed as concentration of subunit using $M_r = 17,000$.

The phosphorylated enzyme ($E - P$) was prepared as described previously (32). The enzyme was first preincubated in T buffer (50 mM Tris-HCl, pH 7.5, containing 5 mM MgCl$_2$ and 75 mM KCl) with a saturating amount of [γ-32P]GTP. It was then freed of nucleotides by gel filtration on Sephadex G-25. The concentration of the phosphorylated enzyme as well as the absence of nucleotides in the preparation were checked from the absorbance spectrum of the protein. The phosphorylated enzyme was then kept on ice and used within 3 h.

**Binding Studies—**The affinity of ADP and 3′-phosphorylated nucleotides for wild type or mutant enzymes was measured by monitoring the variation of intrinsic fluorescence of the protein upon nucleotide binding. All fluorescence measurements were performed at 20 °C in buffer T on a Photon Technology International (PTI) spectrofluorometer (QuantumMaster (2)). Successive aliquots of a concentrated nucleotide solution were added to a 1-mL enzyme solution (2 μM) under continuous stirring, and the fluorescence was measured at 340 nm with excitation at 297 nm (2-nm excitation slit and 2-nm emission slit). After correction for dilution, data were fit to a hyperbolic ligand-protein curve. In the second assay, phosphorylated NDP kinase ($E - P$) was directly incubated with the 3′-P derivatives (0.5 mM) for 10 min at 20 °C. Separation and quantification of 3′-P derivatives were made as above. Finally, the activity of NDP kinase was also measured using a coupled assay (36). For this, the enzyme (0.4 mM) was incubated at 20 °C with ATP (0.2 or 0.4 mM) in the presence or absence of various amounts of 3′-P derivatives. The reaction was initiated by adding dTDP (0.04 mM), and the activity was monitored by the decrease of absorbance at 540 nm. It was verified that the 3′-P derivatives were not inhibitors of pyruvate kinase by monitoring the coupled enzymes activity with ADP as a substrate in the presence or in the absence of 3′-P nucleotides. The reciprocal rates, plotted as a function of the inhibitor concentration for a given ATP concentration, were linearly fitted, and the value of $K_i$ for the 3′ derivative was derived from the common intercept.

**Crystallization and Structure Analysis of Binary Complexes—**Binary complexes were obtained by soaking PAPS in crystals of Dictyostelium NDP kinase grown in hanging drops under the following conditions: 30–32% polyethylene glycol 550, 50 mM Tris-HCl, pH 8.5, and 20 mM MgCl$_2$ in the reservoir; 5 mg/ml protein, 15–16% polyethylene glycol 550, 50 mM Tris-HCl, pH 8.5, 20 mM MgCl$_2$, and 17 mM 3′-amino-ADP in the drop. These conditions set up for analyzing the binding of 3′-amino-ADP (to be described elsewhere) yield crystals growing in 2 weeks. The crystals are trigonal with three subunits in the asymmetric unit and they are isomorphous to the NDP kinase-ADP-AlF$_3$ complex (37). After crystal growth, the bound nucleotide was exchanged by washing overnight in 34% polyethylene glycol 550, 50 mM Tris-HCl, pH 7.5, 20 mM MgCl$_2$, then soaking in the same buffer in the presence of 60 mM freshly dissolved PAPS.

**Fluorometric Titrations—**The binding of the 3′-P derivatives to NDP kinase was monitored by fluorometric titration. The intrinsic fluorescence of the wild type enzyme was quenched in the presence of PAP and PAPS (Fig. 2, inset). This is in contrast to natural nucleosides diphosphates that have no effect on fluorescence (32, 40). The quenching of fluorescence was used to titrate the enzyme (Fig. 2). The dissociation constants ($K_D$) for PAPS and PAP could be computed after fitting the data to

<table>
<thead>
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<th>Table I: Space group</th>
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<td>$R_{free}(%)^c$</td>
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<td>Torsion angle r.m.s.d</td>
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</table>

$R_{merge}$ was calculated using 10% of the total reflections, which are omitted from the refinement.

$^a$ r.m.s.d, root mean square deviation from ideal values.

RESULTS

**Fluorometric Titrations—**The binding of the 3′-P derivatives to NDP kinase was monitored by fluorometric titration. The intrinsic fluorescence of the wild type enzyme was quenched in the presence of PAP and PAPS (Fig. 2, inset). This is in contrast to natural nucleosides diphosphates that have no effect on fluorescence (32, 40). The quenching of fluorescence was used to titrate the enzyme (Fig. 2). The dissociation constants ($K_D$) for PAPS and PAP could be computed after fitting the data to

**Analysis of the x-ray data and the refinement**

Unique reflections, completeness and $R$ factors refer to the values after using a 2 s cutoff on the reflections.

$R_{merge} = \sum(h) - \langle R(h) \rangle / \langle |S(h)| \rangle$, $R(S) = |S(h) - |F(h)|| / |S(h)||$, $R_{free}$ was calculated using 10% of the total reflections, which are omitted from the refinement.
Inhibition of NDP Kinase by 3'-P Nucleotides

Fluorometric titrations were also performed with the F64W mutant enzyme. This mutant was designed to yield a 10% quenching, and this was used to monitor the binding of ADP, cAMP, PAPS, and PAP to the active site (Table II). Typical dissociation constants and their inhibition constants K_I for wild type and F64W enzymes. The values for selected nucleotides for F64W and wild type NDP kinases and their inhibition constants K_I for wild type enzyme are shown in Table II. The dissociation constants KD of selected nucleotides for F64W and wild type NDP kinases and their inhibition constants K_I for wild type enzyme are shown in Table II.

Next, we tested whether a direct transfer of phosphate could occur from phosphorylated NDP kinase to 3'-P-nucleotides. E - 32P (40 μM) was incubated with various nucleotides (0.5 mM). The mixtures were separated by TLC, and the plates were analyzed with a PhosphorImager. Labeled spots at the top of the picture show the inorganic phosphate due to E - 32P dephosphorylation in the conditions of the experiment. Lanes 1–4, respectively, correspond to 0.5 mM ADP, PAP, PAPS, or no addition.

3'-P analogues are not substrates of NDP kinase. E - 32P (40 μM) was incubated with various nucleotides (0.5 mM). The mixtures were separated by TLC, and the plates were analyzed with a PhosphorImager. Labeled spots at the top of the picture show the inorganic phosphate due to E - 32P dephosphorylation in the conditions of the experiment. Lanes 1–4, respectively, correspond to 0.5 mM ADP, PAP, PAPS, or no addition. Next, we tested whether a direct transfer of phosphate could occur from phosphorylated NDP kinase to 3'-P-nucleotides (Fig. 3). Whereas phosphate transfer to ADP was efficient (lane 1), incubation of the 32P-phosphorylated enzyme with PAP or PAPS did not dephosphorylate the enzyme (lanes 2 and 3). We conclude that 3'-P nucleotides are not substrates for NDP kinase.
To analyze NDP kinase inhibition by various analogues, we measured its activity after a short incubation of the enzyme (0.37 mM) with various amounts of 3′-P nucleotides. The data, represented in Fig. 4 as a Dixon plot, show that PAPS is a competitive inhibitor with respect to ATP. This also applies to PAP (data not shown). The inhibition constants ($K_i$) derived from these experiments (Table II) are very close to the $K_D$ values found by fluorescence titration of the wild type and F64W mutant enzymes. With a $K_i$ of 30 μM, PAPS is the best NDP kinase inhibitor among all nucleotide analogues tested thus far.

**Structure of the PAPS-PAP Complex**—The crystalline NDP kinase-PAPS-PAP complex is isomorphous to the previously determined ADP-AlF$_3$ ternary complex (37) and shows no significant change in the protein moiety. The root mean square movement of main chain atoms is 0.3 Å, compatible with estimated errors in atomic coordinates in a 2.8-Å resolution structure.

The 3′-derivatives bind at the same site as the natural substrate ADP, a slit between the Kpn loop and the hairpin made by the $\alpha_A$ and $\alpha_2$ helices (Fig. 5). The base is in the same position, pointing toward the outside of the protein and stacked between Phe$_{64}$ of the loop connecting $\alpha_A$ and $\alpha_2$ and Val$_{116}$ of the Kpn loop (Table III). However, the ribose and phosphate moieties bind differently in the substrate and the inhibitors. The 5′-pyrophosphate moiety of ADP points toward the imidazole group of the active site His$_{122}$. In the ternary complex, AlF$_3$ fits between the $\beta$-phosphate and the N$\delta$ atom of the imidazole, at the expected position of the $\gamma$-phosphate undergoing transfer from ATP to His$_{122}$. With the 3′-derivatives, it is the 3′-phosphate, rather than the 5′-phosphate, that occupies the position near His$_{122}$, only 1.7 Å from where AlF$_3$ is located in the ternary complex (Fig. 6A). Like AlF$_3$ in the ternary complex, the 3′-phosphate makes polar interactions with N$\delta$ of His$_{122}$, the amino group of Lys$_{16}$ and the hydroxyl of Tyr$_{56}$.

The 5′-phosphate points in a direction opposite to that of the 3′-phosphate and toward outside the binding site (Figs. 5 and 6). In subunit A, electron density beyond the 5′-phosphate can be attributed to the PAPS sulfate group in two alternative positions, but there is no equivalent density in subunits B and C. This could be attributed to poor occupancy and/or partial degradation of PAPS yielding PAP. We attempted to refine occupancy by restraining the B factors in the bound nucleotide to a value of 30 Å$^2$. The results suggest that the binding sites are 65–90% occupied by nucleotides in all three subunits, and that each of the two sulfate positions in subunit A has approximately 40% occupancy. The two positions are on the protein surface and only one makes direct interactions with the protein (Table III). In subunits B and C, sulfate occupancy is less than 20%, and the discussion below assumes that the nucleotide is PAP. This difference is accompanied with a difference in sugar puckering, which is 3′-exo for PAPS in subunit A and 2′-endo for PAP in subunits B and C. Bound substrates normally have the sugar in the 3′-endo conformation (24, 25).

A bound Mg$^{2+}$ ion bridges the two phosphates of ADP or dTDP in the binary complexes with NDP kinase (24, 25). In the ADP-AlF$_3$ ternary complex, it makes an additional bond to a fluorine mimicking a $\gamma$-phosphate oxygen. In the present complex, no electron density could be attributed to a metal ion at any of the three binding sites of the asymmetric unit even though 20 mM MgCl$_2$ was present.

**DISCUSSION**

We have identified adenosine derivatives with a phosphate group in the 3′-position which are good competitive inhibitors of NDP kinase. The inhibition constants ($K_i$) for PAP, PAPS, and cAMP derived from measurements of NDP kinase enzymatic activity were compared with equilibrium dissociation constants ($K_D$) obtained by fluorescence titration of the wild type and F64W mutant enzymes. $K_i$ and $K_D$ values were very similar. In addi-
tion, they showed that replacing the Phe$^{64}$ phenyl group with the
indole of a tryptophan has little effect on the nucleotide interac-
tion with the enzyme, even though it is in direct contact with
the nucleobase. The poorest inhibitor is cAMP, with a $K_D$
500
mM. This agrees with a previous kinetic determination (27, 28).
The best is PAPS with a 50 times better affinity.
The crystal structure of the PAPS-PAP complex shows the
3'-phosphate occupying the position expected for the γ-phosphate
of ATP and the 2'-hydroxyl in place of the 3'-hydroxyl of the
substrate. The 2'-hydroxyl H bonds with Lys$^{16}$ and Asn$^{159}$ as
does the 3'-hydroxyl of ADP or dTDP (24, 25). Thus, the 3'-phosphate
group does not disturb the active site conformation.
The replacement of one hydroxyl with the other requires only a
small movement of the whole nucleotide by about 1.5 Å toward
His$^{122}$ (Fig. 6). The 3'-phosphate in PAPS is in contact with
His$^{122}$ and makes polar interactions with Lys$^{16}$ and Tyr$^{56}$. Very
similar interactions are also made by the phosphate moiety of
cAMP bound to Myxococcus xanthus NDP kinase (27). The phos-
phate is also located near the active site histidine, and it H bonds
to residues equivalent to Lys$^{16}$ and Tyr$^{56}$. The cyclic nucleotide
is found to have two conformations, with the base either in syn or
anti position relative to the sugar. The conformation with the base
anti is shown superimposed with PAPS in Fig. 6.
The interactions with $N\delta$ of His$^{122}$, the amino group of Lys$^{16}$,
and the hydroxyl of Tyr$^{56}$ are also made by the fluoride ions of
the ADP-AlF$_3$ ternary complex, and most likely, by the γ-phos-
phate of ATP as a substrate (37). They make major contribu-
tions to catalysis; $N\delta$ is the nucleophile that attacks the
γ-phosphorus, and site-directed mutagenesis has shown that the
deletion of either Lys$^{16}$ or Tyr$^{56}$ side chain decreases activity by
factors of 200 and 50, respectively (34). On this basis, transfer
of phosphate from His$^{122}$ to the 3'-phosphate of PAPS or PAP
could be imagined, but our data indicate that they are not
substrates of NDP kinase. With nucleosides di- or triphos-
phates, phosphate transfer occurs in less than 1 ms as indi-
cated by turnover numbers near 1000 s$^{-1}$. With the 3'-P deriv-
atives, no transfer is observed on a time scale of minutes. Thus,
the 3'-P derivatives are inhibitors, not substrates. The reason

### Table III

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<th>Protein</th>
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<th>PAP (B)</th>
<th>PAP (C)</th>
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<td>N$\xi$ Lys$^{16}$</td>
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<td>3'-Phosphate$^a$</td>
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<td>N$\varepsilon$1 Arg$^{159}$</td>
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<td></td>
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</table>

$^a$ For ADP, this is 3'OH.

$^b$ This is $\beta$-phosphate for ADP.
may be that two major elements of catalysis are missing. (i) One is an H bond within the nucleotide from the 3′-hydroxyl to the oxygen making the bond that is broken or made during transfer. This H bond is specific to NDP kinase and occurs in all complexes with normal substrates. Its deletion in 2′,3′-dideoxynucleotides decreases activity by more than 4 orders of magnitude (1, 2, 40). The acceptor oxygen is the one bridging the β- and γ-phosphorus atoms in ATP and its equivalent would be the 3′-oxygen in PAPS-PAP. (ii) The second is the Mg2⁺ ion. In the ternary ADP-AlF₃ complex, it bridges the two phosphates and the AlF₃ moiety, and must bridge all three phosphates in ATP. No Mg2⁺ was found in the PAPS-PAP complexes.

The 5′-phosphate of bound PAPS-PAP points out from the binding pocket and makes few well defined interactions. The sulfate moiety of PAPS is even less well constrained by the protein environment. It has two alternative positions with similar occupancies, and only one makes direct polar interactions with Arg⁶² and Arg⁹². Yet, PAPS binds better than ATP by an order of magnitude (Table II) corresponding to about 1.4 kcal/mol binding energy. If the direct polar interactions made by the sulfite group in one position were responsible for this difference, the other position should not be observed. A more likely explanation is the generally favorable electrostatic interaction between the negative charge of the sulfate and the many positive charges that surround the NDP kinase nucleotide binding site.

The mode of PAPS-PAP binding observed in the binary complex is suggestive in the context of DNA binding by NDP kinase. Indeed, the human B isoenzyme encoded by nm23-H2 was shown to bind the promoter of the c-myc proto-oncogene and to activate its transcription in vitro (13, 41). Oligonucleotides carrying this sequence or simpler pyrimidine-rich sequences bind both as a duplex and a single strand (15). Participation of the active site in oligonucleotide binding is a possibility, even though it was shown that the active site histidine is not required for DNA binding (42). In the complexes between PAPS-PAP and NDP kinase we see the 3′-phosphate firmly bound near the active site histidine and the 5′-phosphate pointing outward, with the additional sulfite group of PAPS almost free at the protein surface. This sulfite group could be replaced by another nucleoside allowing chain extension in this direction. This suggestion is compatible with mutagenesis studies (43) showing that residues equivalent to Arg⁶⁴, Asn⁶⁹, and Lys⁸⁵ are required for DNA binding.

Are the binding of PAPS-PAP and their inhibition of NDP kinase activity observed here of physiological consequence? PAPS is the major sulfite donor in living cells, and PAP the reaction product. Steady-state concentrations of PAPS in mammalian tissues amount to 10–50 μM and decrease only following a dramatic decrease in ATP (30). 3′-Phosphonucleotides are efficient inhibitors of a variety of cellular enzymes. For instance, 5′-diphosphoasinosine 3′-phosphate was recently found to bind RNAse A very tightly (Kᵢ = 0.2 μM) (44). The crystal structure of a complex of PAP and 17β-estradiol with the estrogen sulfotransferase has been solved (45). The possibility that PAPS or other 3′-P nucleotide analogues could also serve as in vivo NDP kinase inhibitors is attractive. NDP kinase expression is strongly increased in proliferating neoplastic cells and it is possible that its inhibition could affect the growth of a number of tumor cell lines. Cytotoxicity of 3′-P nucleotide derivatives is currently under investigation. Notwithstanding a possible role for PAPS itself, this nucleoside analog can also be considered as a lead compound for future design of high affinity drugs targeted to NDP kinases.

Acknowledgments—We thank Manuel Babalot for excellent technical assistance in kinetics experiments and Dr. Jeff Stock for helpful discussions.
3′-Phosphorylated Nucleotides Are Tight Binding Inhibitors of Nucleoside Diphosphate Kinase Activity
Benoit Schneider, Ying Wu Xu, Joël Janin, Michel Véron and Dominique Deville-Bonne

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