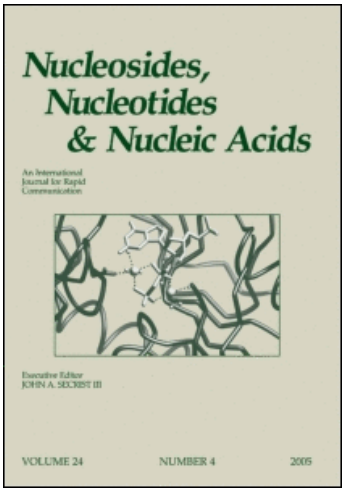

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Striking Ability of Adenosine-2'(3')-deoxy-3'(2')-triphosphates and Related Analogues to Replace ATP as Phosphate Donor for All Four Human, and the *Drosophila Melanogaster*, Deoxyribonucleoside Kinases

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Striking Ability of Adenosine-2′(3′)-deoxy-3′(2′)-triphosphates and Related Analogues to Replace ATP as Phosphate Donor for All Four Human, and the *Drosophila Melanogaster*, Deoxyribonucleoside Kinases

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ABSTRACT

In extension of an earlier report, six non-conventional analogues of ATP, three adenosine-2′-triphosphates (3′-deoxy, 3′-deoxy-3′-fluoro- and 3′-deoxy-3′-fluoroxylo-), and three adenosine-3′-triphosphates (2′-deoxy-, 2′-deoxy-2′-fluoro- and 2′-deoxy-2′-fluoroara-), were compared with ATP as potential phosphate donors for human deoxycytidine kinase (dCK), cytosolic thymidine kinase (TK1), mitochondrial TK2, deoxyguanosine kinase (dGK), and the deoxyribonucleoside kinase (dNK) from *Drosophila melanogaster*. With one group of enzymes, comprising TK1, TK2, dNK and dCK (with dAdo as acceptor), only 3′-deoxyadenosine-2′-triphosphate was an effective donor (5–60% that for ATP), and the other five analogues much less so, or inactive. With a second set, including dCK (dCyd, but not dAdo, as acceptor) and dGK (dGuo as acceptor), known to share high sequence similarity ($\approx$45% sequence identity), all six analogues were good to excellent donors (13–119% that for ATP). With dCK and ATP1, products were shown to be 5′-phosphates. With dCK, donor properties of the analogues were dependent on the nature of the acceptor, as with natural 5′-triphosphate donors. With dCK (dCyd as acceptor), K_m and V_{max} for the two 2′(3′)-deoxyadenosine-3′(2′)-triphosphates are similar to those for ATP. With dGK, K_m values are higher than for ATP, while V_{max} values are comparable. Kinetic studies further demonstrated Michaelis-Menten (non-cooperative) or cooperative kinetics, dependent on the enzyme employed and the nature of the donor. The physiological significance, if any, of the foregoing remains to be elucidated. The overall results are, on the other hand, highly relevant to studies on the modes of interaction of nucleoside kinases with donors and acceptors; and, in particular, to interpretations of the recently reported crystal structures of dGK with bound ATP, of dNK with bound dCyd, and associated modeling studies.

Key Words: Deoxyribonucleoside kinases; ATP analogues; Adenosine-2′(3)′-deoxy-3′(2′)-triphosphates; Donor-acceptor interdependence.

Abbreviations: ATP1, 3′-deoxyadenosine-2′-triphosphate; ATP2, 2′-deoxyadenosine-3′-triphosphate; ATP3, 3′-deoxy-3′-fluoroadenosine-2′-triphosphate (**16**); ATP4, 2′-deoxy-2′-fluoroadenosine-3′-triphosphate (**14**); ATP5, 9-(3-deoxy-3-fluoro-β-D-xylofuranosyl)adenine-2′-triphosphate (**15**); ATP6, 9-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)adenine-3′-triphosphate (**13**); N, nucleoside; NTP, nucleoside 5′-triphosphate; dNK, deoxyribonucleoside kinase from *Drosophila melanogaster*; dCK, deoxycytidine kinase; dGK, deoxyguanosine kinase; TK1, cytosolic thymidine kinase (low-affinity form); TK2, mitochondrial thymidine kinase; HMDS, hexamethyldisilazane; MeOH, methanol; TMS, tetramethylsilane; TMS-Tfl, trimethylsilyl trifluoromethanesulfonate; DAST, (diethylamino)sulfur trifluoride.

INTRODUCTION

Deoxyribonucleoside kinases, which play a key role in the salvage pathway for synthesis of the deoxyribonucleotide precursors required for DNA synthesis, catalyze the following reaction: 2′-deoxyribonucleoside + ATP $\rightarrow$ 2′-deoxyribonucleoside-5′-phosphate + ADP.

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Mammalian cells posses four deoxyribonucleoside kinases, *viz*. cytosolic deoxycytidine kinase dCK (EC 2.7.1.74), cytosolic thymidine kinase TK1, mitochondrial thymidine kinase TK2 (both EC 2.7.1.21), and mitochondrial deoxyguanosine kinase dGK (EC 2.7.1.113).[1] Two of these, dCK and dGK, exhibit overlapping substrate specificities towards dAdo and dGuo, whereas dCK and TK2 both phosphorylate dCyd, and TK1 is highly specific for dThd and dUrd. Other organisms usually have a different number of kinases, often with different and/or overlapping specificities, e.g., the presumed unique deoxyribonucleoside kinase dNK (EC 2.7.1.145) from *Drosophila melanogaster*, which is capable of phosphorylating all four natural deoxyribonucleosides, albeit to highly varying degrees.[2]

The partially overlapping specificities of these enzymes for acceptor substrates with different base and sugar moieties, including many nucleoside analogues which must undergo phosphorylation for expression of antitumour or antiviral activities,[1,3–5] points to considerable flexibility in the active centers of these enzymes. Furthermore, it has long been known that the ATP donor may frequently be replaced by other 5′-NTPs.[6] A striking example is the very marked preference of mammalian deoxycytidine kinase (dCK), and of a bacterial (*B. subtilis*) deoxyguanosine kinase (dGK) for UTP relative to ATP.[7–10]

The foregoing led us to consider whether the location of the triphosphate group of the donor is an absolute requirement for phosphate donor activity. In this context, our attention was directed to a long-overlooked report of Kornberg and coworkers[11] demonstrating that, although deoxyribonucleoside 3′-triphosphates are not substrates for *E. coli* DNA polymerase, their affinities for the enzyme (in the absence of template) are comparable to those of the deoxyribonucleoside-5′-triphosphates; and, indeed, they compete with the latter for the 5′-NTP binding sites. Subsequently Sahyoun et al.[12] isolated from amphibian and mammalian cells a naturally occurring specific inhibitor of adenylyl cyclase, identified as 2′-deoxyadenosine-3′-phosphate. This was followed by numerous reports describing the inhibition of diverse enzymes by nucleotides bearing 3′-phosphate and 3′-polyphosphate groups.[13–15]

We have previously shown that 2′-deoxyadenosine-3′-triphosphate (ATP1) and 3′-deoxyadenosine-2′-triphosphate (ATP2) are relatively good donors for highly purified mammalian dCK, and somewhat less effective with TK1 and TK2.[16] We have now extended these studies to the mammalian deoxyguanosine kinase (dGK), and the deoxyribonucleoside kinase (dNK) from *Drosophila melanogaster*. We have also examined the potential donor properties of four additional 3′(2′)-triphosphates of 2′(3′)-deoxyadenosines with fluorine-substituted pentose moieties, *viz*. ATP3 (**16**), ATP4 (**14**), ATP5 (**15**) and ATP6 (**13**), shown further below in Sch. 2.

EXPERIMENTAL PROCEDURES

Materials

[5-^{3}H]-2′deoxycytidine and [6-^{3}H]-thymidine were from Amersham (UK), and [2,8-^{3}H]-2′-deoxyadenosine and [8-^{3}H]-2′-deoxyguanosine were from Moravek Biochemicals (USA). Non-labelled nucleosides, ATP, DTT, BSA, Tris buffer and inorganic salts were from Sigma (USA).

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Enzymes

All deoxyribonucleoside kinases were recombinants, and procedures for cloning and purification have been previously described, as follows: dCK,[17] dGK,[18,19] TK1,[20] TK2[21] and dNK.[22] The activities of the individual enzymes with standard substrates, determined with ATP as donor, are listed in footnote **b** to Table 4, below.

TK1 may exist in so-called low- (dimer) and high- (tetramer) thymidine-affinity forms, the latter obtained by incubation of the concentrated enzyme with ATP.[23,24] This study is limited to the low-affinity form of TK1, to allow testing ATP analogues in the absence of ATP.

The preparation of dGK contains 0.1 mM ATP (added for purposes of stability). Since this interferes with donor analogue assays, a simple procedure, using 1-mL syringe columns of Sephadex G50, was used to remove ATP. Prior to kinetic experiments, 100 μL aliquots of the enzyme were eluted through these columns by centrifugation ($800 \times g$, 4 min), repeated three times to lower background ATP 3-fold, an acceptable level.

Enzyme Assays

Activities of all kinases were followed at 37°C by a radiochemical assay with the use of ^{3}H-labelled nucleosides, and DE-81 cellulose discs for product separation, essentially as described by Ives et al.[25] Reactions were followed in 50 mM Tris-HCl buffer pH 7.6, containing equimolar amounts of $MgCl_2$ and phosphate donor (ATP or ATP analogue) at 1 mM for activity measurements, or variable for kinetic measurements, and 0.5 mg/mL BSA, 10 mM DTT, indicated concentrations of ^{3}H-labeled 2′-deoxynucleoside, and 0.75–200 ng of the appropriate kinase in a total volume of 50 μL. In each case, the level of enzyme was selected to obtain not more than 20% (usually lower) substrate consumption during ≈30 min incubation, with constant reaction rate. During the course of incubation, 10 μL samples were removed at 8, 16, 24 and 32 min and spotted on DE-81 discs. These were washed three times for 5 min with 5 mM ammonium formate, once in water, then dried and transferred to scintillation vials, containing 0.3 mL 0.1 M HCl/0.2 M KCl. Following elution of the product for 15 min, 2.5 mL liquid scintillator was added, and radioactivity counted. The velocity of a reaction was obtained by least squares linear regression. The activity of a given donor is expressed as the ratio of the velocity of the reaction to that with ATP. Kinetic parameters were determined by the initial velocity method, using non-linear regression fits of the Michaelis-Menten $\{\nu = V_{max} \times [S]/(K_m + [S])\}$ or Hill $\{\nu = V_{max} \times [S]^h/({K_m}^h + [S]^h)\}$ equations. For control (background) activity, the reaction medium contained all components, except the phosphate donor, and usually, with the exception of dGK (see above), showed no detectable activity.

Site(s) of Phosphorylation

Although deoxyribonucleoside kinases, with a 5′-NTP as phosphate donor, are known to phosphorylate only the primary 5′-hydroxyl of a nucleoside acceptor, it was considered necessary to check whether this is also the case with our

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deoxynucleoside-3′(2′)-triphosphate donors. Hence products of phosphorylation were treated at pH 7.5 with Russell's Viper venom, a rich source of 5′-nucleotidase, verified as totally inactive against nucleoside-2′(3′)-phosphates, and dephosphorylation monitored by disappearance of radiolabeled product on DE-81 disks. An interesting, and fortunate, finding was that the venom 5′-nucleotidase, although inhibited by ATP, was not by ATP1.

Synthetic Procedures

General

UV spectra were recorded on a Specord M-400 (Carl-Zeiss, Germany). ^{1}H NMR spectra were run at 200.13 MHz at 23°C on an AC-200 spectrometer equipped with an Aspect 3000 data system (Bruker, Germany) with TMS as internal standard (s = singlet, d = doublet, t = triplet, m = multiplet, br.s = broad signal). Assignment of proton resonances was confirmed, where possible, by selective homonuclear decoupling. Procedures for TLC and HPLC are given in footnotes to Table 2. Flash silica gel column chromatography of nucleosides was performed on 230–400 mesh silica gel (Merck, Germany). In condensation reactions, freshly distilled trimethylsilyl trifluoromethanesulfonate (TMS-Tfl, Fluka, Switzerland) was employed. Solutions of compounds in organic solvents were dried with anhydrous sodium sulfate for 4 h. Unless otherwise indicated, reactions were carried out at 20°C.

Condensation of 1-*O*-acetyl-2-deoxy-2-fluoro-3,5-di-*O*-benzoyl-D-arabinofuranose (2) with Silylated Adenine (1). A solution of arabinoside **2** (0.25 g, 0.62 mmol), trimethylsilyl derivative of adenine (**1**) [obtained from adenine (90 mg, 0.66 mmol) by refluxing in HMDS (0.7 mL) in the presence of TMS-Cl (0.1 mL)] and TMS-Tfl (0.3 mL, 1.8 mmol) in anh 1,2-dichloroethane (3 mL) was stirred at r.t. for 48 h, poured into 5% aq. solution of $NaHCO_3$ (10 mL) and extracted with $CHCl_3$ (3 × 15 mL). The combined organic extracts were evaporated, the residue was treated with MeOH (15 mL), saturated with dry ammonia gas at 0°C, for 20 h and evaporated. The residue was chromatographed [silica gel column, 5 × 40 cm; elution with a linear MeOH gradient in $CHCl_3$ (0 → 10%, v/v; 2 × 300 mL)] to yield the N^9-β-isomer **3** (35 mg, 21%) and the N^9-α-isomer **4** (18 mg, 11%; amorphous). Compounds **3** and **4** are identical in all respects (^{1}H NMR and UV spectra, TLC and HPLC) to those previously prepared.[26]

9-(2-Deoxy-2-fluoro-β-D-arabinofuranosyl)adenine (3). Compound **5** (1.12 g, 1.5 mmol)[3] was added to a solution of DAST (1.3 mL, 10.0 mmol) in a mixture of anh. CH_2Cl_2 (20 mL) and pyridine (1.6 mL), the reaction mixture was heated at 50°C for 3 h, poured into 5% aq. solution of $NaHCO_3$ (20 mL) and extracted with $CHCl_3$ (3 × 25 mL). The combined organic extracts were evaporated, the residue was treated with MeOH (15 mL), saturated with dry ammonia gas at 0°C, for 20 h and evaporated. The residue was dissolved in 80% aq. solution of CH_3COOH

(10 mL), the mixture was stirred for 1 h and evaporated. The residue was chromatographed as described above to yield the N^9-β-isomer **3** (40 mg, 10%).

Phosphorylation

The nucleosides **9–12** were converted to the corresponding triphosphates **13–16** (Sch. 2), as previously described for the preparation of ATP1 and ATP2 in Ref.[16], with appropriate data given in Table 2.

RESULTS AND DISCUSSION

Syntheses

We have previously demonstrated that the coupling of trimetylsilylated N^6-benzoyladenine with 1,3,5-tri-O-benzoyl-2-deoxy-2-fluoro-β-D-ribofuranose in the presence of an excess of TMS-Tfl in anhydrous acetonitrile under reflux led to a mixture of isomeric benzoylated nucleosides. Debenzoylation of this mixture followed by double silica gel column chromatography permitted isolation of pure nucleosides 9-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)adenine (**3**; 14%), it's α-anomer (14%), and N^7-α-arabinoside (25%).[26] We have now employed trimetylsilylated adenine (**1**) in the reaction with 1-O-acetyl-3,5-di-O-benzoyl-2-deoxy-2-fluoro-β-D-ribofuranose (**2**) for the preparation of arabinoside **3** (for a recent review on nucleoside synthesis, see[27]). The condensation of **1** with **2** in the presence of excess TMS-Tfl in anhydrous dichloroethane at room temperature, followed by conventional workup of the reaction mixture, deprotection of products and silica gel column chromatography, afforded arabinoside **3** and it's α-anomer **4** in 21% and 11% yields, respectively (Sch. 1).

We have also studied an alternative synthetic route to arabinoside **3**, which employs the readily accessible N^6,$O^{3'}$-dibenzoyl-5′-*O*-(*p*-monomethoxy)trityladenosine (**5**)[28] as starting compound. Treatment of **5** with DAST in CH_2Cl_2 in the presence of pyridine at 50°C for 3 h, followed by workup of the reaction mixture, deprotection of products and silica gel column chromatography, gave arabinoside **3** in 10% yield (Sch. 1). The yield of the desired arabinoside **3** in this reaction was not optimized. It was recently shown that the treatment of 9-(3-*O*-benzoyl-5-*O*-trityl-β-D-ribofuranosyl)-6-chloropurine with DAST under similar reaction conditions resulted in the formation of the 2′-deoxy-2′-fluoro-arabinosyl derivative in 78% yield.[29] Thus, the transformation of **5** into **3** deserves more detailed investigation as a reasonable alternative to the convergent approach.

The synthesis of 2′-deoxy-2′-fluoroadenosine (**6**), 9-(3-deoxy-3-fluoro-β-D-xylofuranosyl) adenine (**7**) and 3′-deoxy-3′-fluoroadenosine (**8**) was previously described in Ref.[28,30–32] Compounds **3, 6–8** were transformed into the respective derivatives **9–10** as reported previously [16,28 and references cited] (Sch. 2).

The ^{1}H NMR and other physico-chemical data for compounds **11** and **12** have been described in Ref.[28] Similar data for compounds **9** and **10** are listed in Tables 1 and 2.

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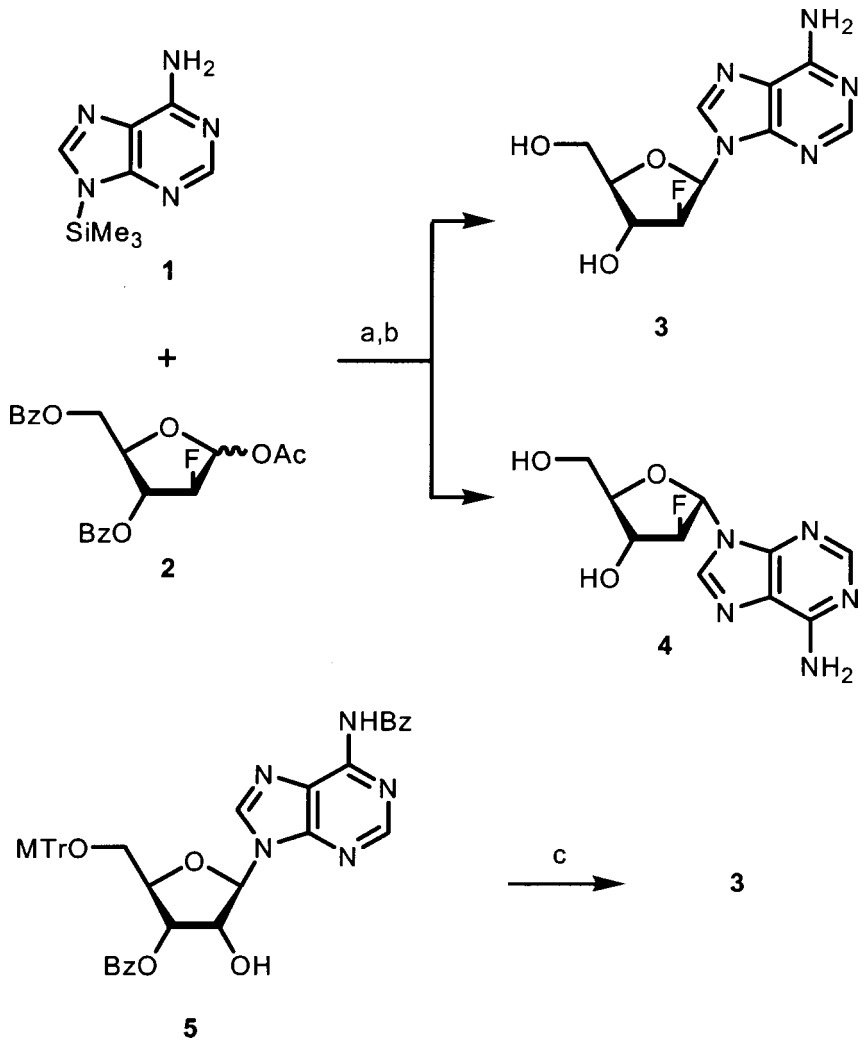
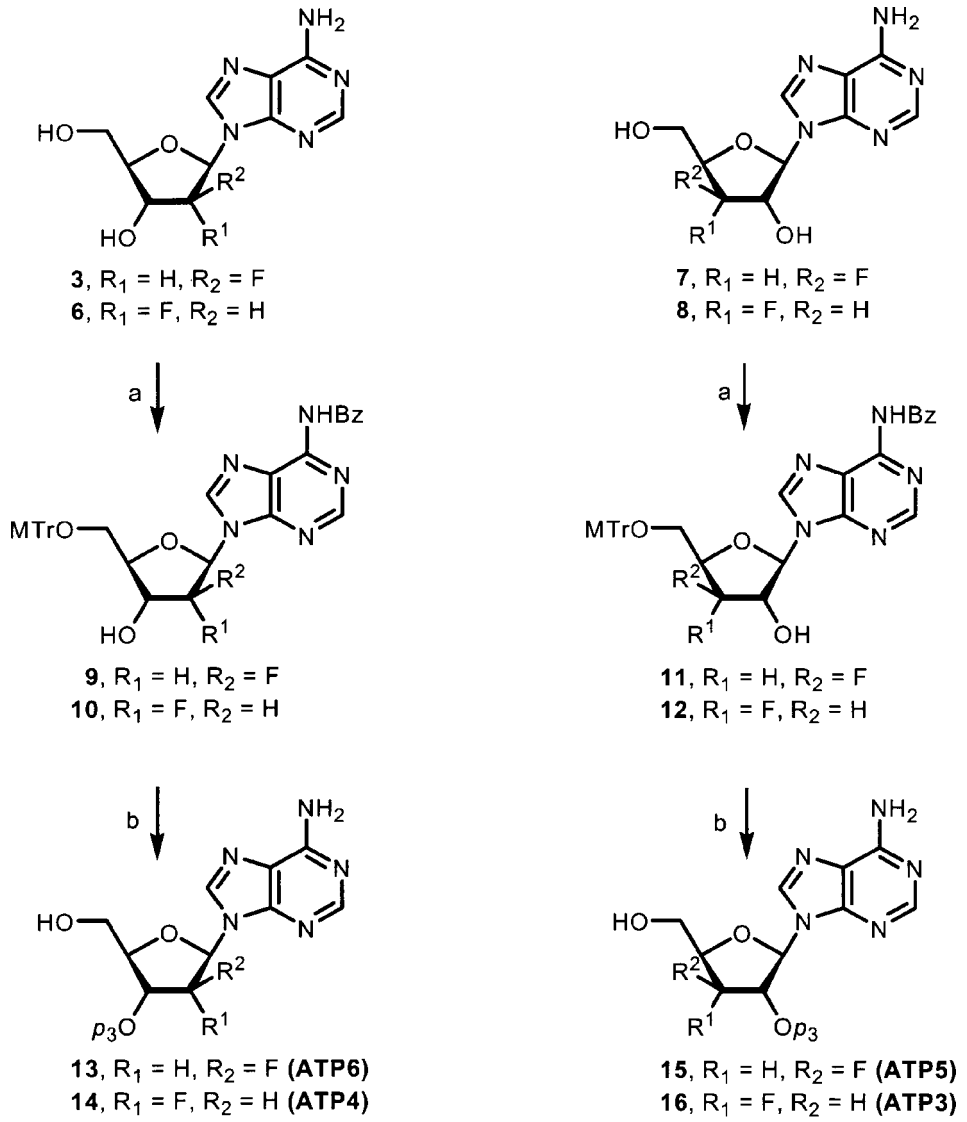


Scheme 1. (a) **1**/**2**/TMS-Tf1 (1.1:1.0:3.0, mol), CH_2ClCH_2Cl, room temperature, 48 h; silica gel column chromatography; (b) NH_3/MeOH, room temperature, 48 h; silica gel column chromatography (combined yields: **3**, 21%; **4**, 11%); (c) *1*-DAST/Py (1.0:2.0, mol), CH_2Cl_2, 50°C, 3 h; *2*-NH_3OH/MeOH, room temperature, 20 h; 3-80% aq. CH_3COOH, room temperature, 1 h (**3**, Σ10%).

The triphosphates **13** (ATP6), **14** (ATP4), **15** (ATP5), **16** (ATP3) were then prepared as described previously,[16,28], as shown in Sch. 2. Relevant physico-chemical data are presented in Table 2.

Conformational Analysis of the Furanose Rings

This was performed in order to reveal some possible correlations between the stereochemistry of the furanose rings and the donor activities of ATP analogues



Scheme 2. (a) 1-TMS-C1/Py; 2-BzCl; NH_4OH; *3*-MTr-Cl/Py (Σ65–75%); (b) 1-1M solution of $PO(Im)_3$ in anh. pyridine, r.t., 4–6 h; 2-$P_2O_7H_2^{2-}$ x$(Bu_3HN^{1+})_2$, DMF/Bu_3N, r.t., 30 min; 3-0.5 M TEAB buffer, r.t., 3 h; 4-conc. NH_4OH, r.t., 20 h; 5-80% aq. CH_3COOH, r.t., 1 h: 6-DEAE Sephadex [A-25, HCO_3^{1-}, linear TEAB gradient (0.001–1 M, 2×500 mL)], (Σ32–43%).

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Table 1. ^{1}H NMR data for 9-[2-deoxy-2-fluoro-5-*O*-(*p*-monomethoxy)trityl-β-D-arabinofuranosyl]-N^{6}-benzoyladenine (**9**) and its *ribo*-isomer **10** in $CDCl_3$.*

	Chemical shifts, δ_{TMS}, ppm								Coupling constants, Hz								
	Base		Sugar						^{3}J(H, H)					^{5}J(H,F)	^{3}J(H, F)		$^{2}J_{gem}$(H, F)
Compound	H-2	H-8	H-1′	H-2′	H-3′	H-4′	H-5′	H-5″	1′,2′	2′,3′	3′,4′	4′,5′	4′,5″	8,F	1′,F	3′,F	2′,F
9	8.80 s	8.23 d	6.63 dd	5.10 ddd	4.63 dm	4.15 m	3.52 dd	3.42 dd	2.9	≈2.5	≈3.0	5.5	5.5	2.6	19.5	18.0	52.0
10	8.76 s	8.22 s	6.29 dd	5.66 ddd	4.84 dm	4.23 m	3.57 dd	3.43 dd	2.0	4.0	≈6.0	3.5	4.3	–	16.5	17.0	53.0

*The following resonances in the ^{1}H NMR spectra of **9** and **10** are not included: NH (broad signal) – 9.16 and 9.19 ppm, 3′-OH (broad signal) – both at 2.82 ppm; both at 8.04 ppm (br.d, 2H, $^{3}J_{ortho,meta}$ ≈7.0 Hz, the *ortho* protons of the Bz group); both within 7.20–7.64 ppm (aromatic protons); 6.84 and 6.80 ppm (d, 2H, $^{3}J_{ortho,meta}$ = 9.5 Hz, the *meta* protons of the MTr group); both at 3.80 ppm (s, 3H, OCH_3).

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Table 2. Properties of 9-[2-deoxy-2-fluoro-5-*O*-(*p*-monomethoxy)trityl-β-D-arabinofuranosyl]-N^6-benzoyladenine (**9**) and its *ribo*-isomer **10** and triphosphates **13–16**.

Compound	Combined yield (%)	TLC[a] (R_f value)	UV spectrum (MeOH)		HPLC[b]	
			λ_{max}	ε	R_t (min)	Purity (%)
9	60	0.29	233.0; 280.0	33900; 27600	–	–
10	54	0.32	232.0; 281.0	41500; 23200	–	–
13	40	0.19	260.0	13600	3.34	91
14	43	0.21	260.0	14200	5.63	96
15	32	0.17	260.0	14000	3.93	94
16	43	0.19	260.0	13800	7.47	93

[a]TLC: aluminium sheets silica gel 60 F_{254} (Merck, Germany), solvent systems: 2-propanol/25% aq. ammonia/water, 11 : 7 : 2 (vol).
[b]HPLC: Waters apparatus (Waters, USA); column Nova-Pac C-18 (3.9 × 300 mm)(Waters); isocratic elution with 7% MeCN in 0.1M KH_2PO_4 (v/v), flow rate 0.7 mL/min (time of analysis 15 min).

studied. The PSEUROT (version 6.3) program was employed for the conformational analysis of the furanose rings of 9-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)adenine (**3**), 2′-deoxy-2′-fluoroadenosine (**6**), 9-(3-deoxy-3-fluoro-β-D-xylofuranosyl)adenine (**7**) and 3′-deoxy-3′-fluoroadenosine (**8**). This program calculates the best fits of three ^3J(H, H) and two ^3J(H, F) experimental coupling constants ($^3J_{H1',H2'}$, $^3J_{H2',H3'}$ and $^3J_{H3',H4'}$, as well as $^3J_{H1',F2'}$ and $^3J_{H3',F2'}$ in the case **3** and **6**, and $^3J_{H2',F3'}$ and $^3J_{H4',F3'}$ in the case of **7** and **8**) to the five conformational parameters (P and ψ_m for both the

Table 3. Pseudorotational parameters of 9-(2-deoxy-2fluoro-β-D-arabinofuranosyl)adenine (**3**), 2′-deoxy-2′-fluoroadenosine (**6**), 9-(3-deoxy-3-fluoro-β-D-xylofuranosyl)-adenine (**7**), and 3′-deoxy-3′-fluoroadenosine (**8**) in D_2O solutions.

Compound	P_N	$\psi_{m(N)}$	P_S	$\psi_{m(S)}$	rms	$\lvert\Delta J_{max}\rvert$	%S	$N \leftrightarrow S$ equilibrium
3[a] (J_{HH})	−2.5	40[b]	118.6	40[b]	.132	.21	67	$^3_2T \rightarrow {}_1T^0$
(J_{HH} & J_{HF})	11.6		123.6		.235	.69	66	$^3T_2 \rightarrow {}_1E$
6[c] (J_{HH})	32.5	45[b]	201.6	40[b]	.000	.00	36	$^3_4T \leftarrow {}_3E$
(J_{HH} & J_{HF})	39.3		210.7		.295	.90	40	$^3_4T \leftarrow {}_3T^4$
7[d] (J_{HH})	30.6	32.5	162[b]	39[b]	.000	.00	14	$^3T_4 \leftarrow {}^2E$
(J_{HH} & J_{HF})	31.1	33.4			.093	.28	13	$^3T_4 \leftarrow {}^2E$
8[d] (J_{HH})	10.0[b]	39[b]	160.4	35.1	.000	.00	98	$^3T_2 \rightarrow {}^2E$
(J_{HH} & J_{HF})			158.7	35.0	.050	.17	97	$^3T_2 \rightarrow {}^2E$

[a]Coupling constants from Ref.[26].
[b]Values indicated were fixed during final calculations.
[c]Coupling constants from Ref.[30].
[d]Coupling constants from Ref.[37].

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N- and *S*-type conformers and the corresponding mole fractions).[33] Optimization of pseudorotational parameters was performed essentially as described previously.[34] The resulting optimised geometries of the *N*- and *S*-pseudorotamers are presented in Table 3.

It was previously shown on the basis of ^3J(H, H) analysis that the sugar moiety of the β-arabinoside **3** is in a two-state *N*- and *S*-pseudorotational equilibrium in the ratio 36 : 64[26]. Rather similar results were obtained in the present work (Table 3). Note that, in the predominantly populated $_1E$ (*S*-type) conformation, the F-C(2′)-C(1′)-O(4′) and F-C(2′)-C(1′)-N(9) fragments adopt the *gauche* orientation, whereas the other possible *gauche* interactions [C(2′)-F/ C(3′)-OH and C(3′)-OH and the ring oxygen] are not realised. Furthermore, in the less populated $^3T_2$ (*N*-type) conformation, two *gauche* effects are operative [C(2′)-F/C(3′)-OH and C(2′)-F/C(1′)-N(9)] along with the anomeric effect, which is more optimal in the *N*-type conformation.[35,36] Further support for the $_1\mathrm{E} \leftrightarrow {}^3T_2$ equilibrium results from the observed ^3J(F, C(4′)) coupling constant of 3.39 Hz,[26] which is an average value of the above conformations: in the $_1E$ conformation the C(4′)-C(3′)-C(2′)-F torsion angle is about 95°, whereas in the $^3T_2$ conformation the same torsion angle is about 165°.

In the case of riboside **6**, the HO-C(3′)-C(2′)-F fragment adopts the *gauche* orientation in both *N*- and *S*-type pentofuranose ring conformations. The *gauche* interaction between C(2′)-F and the ring oxygen, along with the anomeric effect, drive the $N \leftrightarrow S$ equilibrium toward the *N*-conformation. Note that the two aforementioned effects slightly predominate over the *gauche* effect of HO-C(3′)-C(4′)-O(4′). It is noteworthy that ^3J(F, C(4′)) was found to be < 2.0 Hz, consistent with a predominant $^3_4$T conformation (the C(4′)-C(3′)-C(2′)-F torsion angle is around 90°) as distinct from the less populated $_3T^4$, where the same torsion angle is around 165°.

There is very good concordance of results from the previous PSEUROT analysis of ^3J(H, H) of the furanose rings of the 3′-fluoro substituted nucleosides **7** and **8**[37] and those herein presented, employing both ^3J(H, H) and ^3J(H, F) coupling constants. The pentofuranose ring of the xyloside **7** assumes the most populated $^3T_4$(*N*-type) conformation, mainly by virtue of the *gauche* effect of F-C(3′)-C(4′)-O(4′). Moreover, the anomeric effect drives the $N \leftrightarrow S$ equilibrium toward the *N*-conformation. In the case of riboside **8**, like the isomeric riboside **6**, the HO-C(2′)-C(3′)-F fragment adopts the *gauche* orientation in both *N*- and *S*-type conformations. Therefore, the *gauche* interaction between C(3′)-F and C(4′)-O(4′) bonds is the predominant factor leading to the dominant population of the ^2E (*S*-type) conformation.

The values of the vicinal $^3J_{\mathrm{H(1'),C(8)}} = 4.55$ Hz and $^3J_{\mathrm{H(1'),C(4)}} < 2.0$ Hz couplings of the arabinoside **3** unambiguously point to the predominant *anti*-conformation about the glycosidic bond.[26,38,39] By contrast, the isomeric riboside **6** reveals higher conformational mobility about the glycosidic bond, reflected in identical values of the aforementioned vicinal coupling constants, equal to 3.80 Hz. The predominant *anti*-conformation of the adenine bases of the 3′-fluorinated nucleosides **7** and **8** was previously deduced from the spin-lattice relaxation times of H(8) and H(1′).[37]

One may expect that introduction of a triphosphate group at the secondary hydroxyl of nucleosides **3, 6–8** should not lead to essential changes of the $N \leftrightarrow S$

conformational equilibrium of the pentofuranose rings. The *cis*-arrangement of the fluorine atom and the phosphate group does not result either in effective steric repulsion, or in additional stabilisation of any conformation, by means of hydrogen bonding. It was, however, shown that a 3′-phosphate group in ribonucleosides drives the $N \leftrightarrow S$ pseudorotational equilibrium somewhat more to the S-type pseudorotamers vs. the 3′-hydroxyl, implying a stronger *gauche* effect of O(4′)-C(4′)-C(3′)-O-phosphate than of the O(4′)-C(4′)-C(3′)-OH fragment.[16,38] Concurrently, the enhanced ability of the former fragment to influence the $N \leftrightarrow S$ pseudorotational equilibria was shown to be lower in the case of purine compounds. In toto, there are no evident reasons to expect noticeable changes in conformation on going from nucleosides **3, 6–8** to the corresponding triphosphates **13–16**.

Enzymatic Aspects

It should be noted that, with human dCK, it is now well documented that UTP is a much more effective donor than ATP in vitro and, to a considerable extent, in vivo.[8–10] But, since our 2′(3′) -triphosphates are analogues of ATP, the latter is employed throughout as the reference donor.

The phosphate donor activities of all six ATP analogues, expressed as initial rates of phosphorylation relative to that for ATP (taken as 100%), are shown in Table 4. The major substrates of TK1 (dThd) and dGK (dGuo) were used as phosphate acceptors, while two substrates were used with dCK (dCyd and dAdo), TK2 (dThd and dCyd) and dNK (dCyd and dAdo). With dCK and TK2 the second substrate (dAdo and dCyd, respectively) is also effectively phosphorylated, but differs significantly in kinetic parameters from the major one. For dNK all four natural 2′-deoxynucleosides are substrates,[2] leading to selection of one pyrimidine (dCyd) and one purine (dAdo) acceptors. Footnote **b** to Table 4 lists the actual activities of ATP with the different enzymes for the substrates employed, each at a concentration exceeding its K_m value.

It should be noted that the nucleoside acceptor concentrations used here (Table 4) are relatively high as compared with their physiological concentrations (see[8] and references cited therein). Hence phosphorylating activities of the enzymes may not reflect the situation under physiological conditions. Concentrations of substrates used here were dictated by the need for substrate saturation, usually achieved in a concentration range 10-fold higher than the appropriate K_m values. Consequently, initial rates measured here may be regarded as relatively good approximations of V_{max} values, hence also of the turnover rates.

Site(s) of phosphorylation were first established by treatment of phosphorylated products with 5′-nucleotidase, as described under Experimental Procedures. With dCK, and ATP1 as donor, phosphorylation of dCyd and dAdo led only to nucleoside-5′-phosphates, other enzymes and phosphate donor analogues were not tested.

Quite striking is the fact that, with the enzyme dCK, and dCyd as acceptor, all analogues exhibited donor activities comparable to that of ATP, with ATP1 as fully active as ATP. However, with dAdo as acceptor, only ATP1 exhibited high activity, 60% that for ATP, the rates for all the others being below 10% that for ATP. Equally striking is the finding that, with the enzyme dGK, ATP1 is even more active than

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Table 4. Phosphate donor activities of ATP analogues (all at 1 mM) towards deoxyribonucleoside kinases,[a] expressed as initial rates relative to that of ATP (also at 1 mM, taken as 100%[b]) and with different acceptors at concentrations indicated.

		dCK			TK1	TK2		dNK		dGK[c]
% of donor N-conformer	Donor	dCyd 2 μM	dCyd 25 μM	dAdo 50 μM	dThd 30 μM	dThd 1 μM	dCyd 50 μM	dCyd 2.5 μM	dAdo 100 μM	dGuo 10 μM
≈50	ATP	**100**	**100**	100	100	100	100	100	100	**100**
≫50	ATP1	**84**	**97**	60	5.3	7	15	10	6	**119**
≈50	ATP2	**52**	**73**	6	2.3	2.2	1.6	1	0.5	**53**
3	ATP3	**58**	**66**	4	0	1.7	1.7	0.2	0	**25**
65	ATP4	**36**	**62**	1.5	1	1.0	1.2	0.6	0.2	**70**
85	ATP5	**59**	**62**	4	0	0.9	0.7	0	0	**55**
35	ATP6	**38**	**59**	1	0	0.6	0.2	0	0	**13**

[a]dCK (with dAdo as acceptor), TK1, TK2 and dNK, tentatively classified as group (a); dCK (with dCyd as acceptor) and dGK (numbers in bold) as group (b), as described in the text.
[b]Activities of enzymes with ATP are as follows:
dCK/2 μM dCyd – 21 nmol/min/mg
dCK/25 μM dCyd – 27 nmol/min/mg
dCK/50 μM dAdo – 540 nmol/min/mg
TK2/1 μM dThd – 130 nmol/min/mg
TK2/50 μM dCyd – 520 nmol/min/mg
TK1/30 μM dThd – 330 nmol/min/mg
dNK/2.5 μM dCyd – 3.0 μmol/min/mg
dNK/100 μM dAdo – 2.70 μmol/min/mg
dGK/10 μM dGuo – 23 nmol/min/mg
[c]1% background activity (in absence of donor) due to contamination of dGK with ATP.

ATP, and that ATP2, ATP4 and ATP5 each exhibits activity at a level exceeding 50% that for ATP. Even ATP3 (25% activity) and ATP6 (13% activity) can be considered as reasonably good donors. It is consequently worth noting that, amongst all five enzymes, dCK and dGK are known to exhibit highest sequence similarity, with about 45% identity.[40]

With the other three enzymes, only ATP1 exhibits moderate donor activity, 5–15% that for ATP, whereas all the other analogues are only marginally (2%) active, or not at all. Note that, with TK2, ATP1 is 2-fold more active vs. dCyd as compared to dThd; and with dNK, it is more active vs. dCyd than dAdo. It should be noted that TK2 and dNK, with similar donor specificities, also exhibit high (40%) sequence identity.[40]

Overall, ATP1 is the most effective donor for all five kinases, whereas ATP2-ATP6 together may range from excellent to neglible activity, depending on the kinase employed, so that the enzymes (or, more precisely, enzyme activities) may be tentatively classified in two groups: (a) TK1, TK2, dNK and dCK (but only with dAdo as acceptor), with restricted donor specificity, are significantly active only with ATP1; (b) dCK (with dCyd as acceptor) and dGK, with relaxed donor specificity, which accept all six donor analogues at a level comparable with, or even superior

to, that for ATP (cf. columns in bold and non-bold in Table 4). We revert to this further, below.

The conformational parameters of our ATP analogues, considered to be similar to those of the corresponding parent nucleosides (see last paragraph of Conformational analysis), exhibit marked variations, particularly as regards the $N \leftrightarrow S$ equilibria, and the puckering modes of the furanose rings (Table 3). But, as may be seen from Table 4, there is no apparent satisfactory correlation between these parameters and donor activities. Presumably the enzyme constrains a bound donor to the conformation required. It should, nonetheless, be noted that, in the case of dGK, the $N \leftrightarrow S$ equilibrium of the pentafuranose rings does appear to influence phosphate donor activities. Furthermore, amongst the enzymes in group (a), the presence of an electronegative fluorine at the pentose C(2′) or C(3′) does lead to a marked decrease in donor activities of ATP3-ATP6 relative to their non-fluorinated analogues ATP1 and ATP2.

Kinetic parameters for two of the analogues, ATP1 and ATP2, as compared to those for ATP, are presented in Table 5. It was previously shown that, with native dCK, both ATP1 and ATP2 were competitive with respect to ATP.[16] It will be seen that, with dCK, both K_m and V_{max} values are comparable for all three donors, with 25 μM dCyd as acceptor, in line with their rates of phosphorylation (see Table 4). With TK1, K_m values are also comparable, but V_{max} values are much lower for ATP1 and ATP2, in accord with their much poorer abilities as donors (Table 4).

Table 5. Kinetic parameters for the phosphate donors ATP1 and ATP2, and ATP for comparison, with various deoxyribonucleoside kinases. V_{max} is expressed as % reaction rate with 1 mM ATP (see Table 4, footnote b), and h is the Hill coefficient. Acceptors employed, and their concentrations, are listed for each kinase

Donor \ Enzyme		dCK (25 μM dCyd)	TK1 (30 μM Thd)	TK2 (1 μM Thd)	dNK (10 μM dCyd)	dGK (20 μM dGuo)
ATP	K_m^{app} (μM)	7 ± 1 (60 ± 7)[a]	140[b]	2[c]	1.4[d]	20 ± 3[e]
	V_{max} (%)	103 (106)[a]	104[f]	100[f]	100[f]	104
	h	0.7 (1.0)[a]	1.6[b]	1.0[c]	1.0[d]	0.8
ATP1	K_m^{app} (μM)	11 ± 1.5	116 ± 37	15 ± 4	24 ± 1	no saturation up to 4 mM
	V_{max} (%)	98.7	5.9	7.1	18.6	
	h	0.8–1.0	(1.0)[g]	1.0	1.3	0.5–0.6
ATP2	K_m^{app} (μM)	7.4 ± 0.3	165 ± 20	36 ± 13	28.5 ± 3.5	130 ± 67
	V_{max} (%)	73.5	2.7	2.2	1.6	67
	h	1.0	(1.0)[g]	0.7	0.8	0.75

[a]Values in brackets are for 50 μM dAdo as acceptor.
[b]Data from.[24]
[c]Data from.[57]
[d]Data from.[2]
[e]A much lower value, 2.8 ± 0.5 μM, was reported by Herrström Sjöberg et al.[58]
[f]Calculated using published K_m values (see footnotes b-d) and reaction rates with 1 mM ATP.
[g]Too few data points to establish kinetic model, but kinetics appear hyperbolic.

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By contrast, with TK2 and dNK (sequence identity $\approx 40\%$), K_m values for both ATP1 and ATP2 are much higher than for ATP.

Effects of Acceptor on Kinetics of Donor

Studies on nucleoside kinases have hitherto overlooked the possible influence of a change of acceptor on the kinetics of the traditional ATP (or other 5′-NTP) donor. Our findings with nucleoside-2′(3′)-triphosphate donors clearly demonstrate that a change of acceptor may appreciably affect the donor specificity, as shown for dCK in Table 4, prompting us to examine the kinetic parameters for ATP with dCK, using dAdo as acceptor in place of dCyd. This led to a change not only in kinetics, cooperative with dCyd ($h = 0.7$), and non-cooperative with dAdo ($h \approx 1$), but also to a marked increase in K_m for ATP from $7 \pm 1\,\mu M$ (with dCyd) to $60 \pm 7\,\mu M$ (with dAdo), while its V_{max}/K_m was still higher (≈ 3-fold) with dAdo as acceptor than with dCyd, due to the much higher turnover with dAdo (Table 5; Table 4, footnote b). The ratio V_{max}/K_m for dCyd is 20-fold higher than for dAdo,[41] so that the difference in V_{max}/K_m between the two acceptors at fixed (saturated) ATP concentration is much higher. Nonetheless, this is the largest reported modification in donor kinetics caused by a change of phosphate acceptor.

Furthermore, whereas activity of dATP with dCK (dCyd as acceptor), is only $\approx 60\%$ that for ATP, and GTP, dGTP and dTTP are as effective as ATP, replacement of dCyd by dAdo led to a marked decrease in activity of the donor, 2-3-fold with dGTP and dTTP, and as much as 30-fold with dATP. The foregoing underlines the broad substrate specificity with respect to donors for dCK (with dCyd as acceptor), but much less so with the group (a) enzymes (activities); and is particularly well reflected in the case of dCK (with dAdo as acceptor), showing that a change of acceptor may affect the range of specificity of the enzyme with respect to the donor.

Relevant to the foregoing are our earlier results on dependence of the relative phosphate uptake form the ATP component of an ATP:UTP mixture, catalyzed by dCK, on the phosphate acceptor,[8] demonstrating a remarkable preference for UTP, relative to ATP, and applicable with both dCyd and dAdo as acceptors. It would be interesting, in this context, to determine if 2′(3′)-deoxy-3′(2′)-triphosphates of uridine behave like the adenosine analogues tested here, particularly as regards dependence on phosphate acceptors.

Biological Aspects

The ability of deoxyadenosine -2′- and 3′-triphosphates to replace ATP as phosphate donors with some deoxyribonucleoside kinases clearly calls for a reexamination of current concepts on the mode of binding, and the mechanism of action, of ATP as a phosphate donor not only for nucleoside kinases, but also for other kinase systems, e.g., protein kinases [see, e.g., 6,42]. In this context, it is worth noting that numerous reports describe nucleoside-2′(3′)-phosphates and 3′-polyphosphates as potent enzyme inhibitors. A naturally occurring inhibitor of adenylyl cyclase, long ago isolated from membranes of amphibian and mammalian cells, was identified as 2′-deoxyadenosine-3′-phosphate.[12,43] Subsequently synthetic adenine nucleoside 3′-polyphosphates were

found to be even better inhibitors of such enzymes.[44] The two most potent inhibitors of RNase A hitherto reported are the 2′- and 3′-phosphates of 5′-diphosphoadenosine.[14] The naturally occurring 3′-phosphates of 5′-phosphoadenosine (PAP) and of adenosine-5′-phosphosulfate (PAPS), both tight-binding inhibitors of nucleoside diphosphate kinases, have been proposed as high-affinity drugs targeted to these enzymes.[13] The natural occurrence and formation of nucleoside-3′-phosphates, resulting from enzymatic degradation of oligo and polynucleotides by tissue extracts, has been reported by Bushfield et al.[45] We are, however, not aware of any reports on the natural occurrence of nucleoside-2′-phosphates, which might be anticipated as products of catabolism of the interferon-induced 2′-5′ oligoadenylates.[46]

It has long been known that ATP may be replaced as a phosphate donor by other 5′-NTPs in various kinase systems,[6,42] including viral TKs.[3,4] For example, UTP is a far superior donor than ATP with human dCK,[8] and is 50-fold more effective than ATP for the dGK from *B. subtilis*.[7] CTP is a good donor with vertebrate mitochondrial TK2,[1] and as efficient as ATP with dNK.[2,22] Probably the most striking example is bovine mitochondrial dGK,[47] for which ATP is the best donor at its optimum pH, 5.5, whereas at physiological pH its activity is drastically decreased, while CTP and dCTP were as active as ATP, and UTP and dTTP twice as active.

The known broad specificity of bovine dGK for natural 5′-NTP donors[47] is reflected here by the broad specificity for 2′-, 3′- and 5′-triphosphate donors of human dGK (with dGuo as acceptor) and dCK (with dCyd as acceptor). With TK1, TK2 (specific for pyrimidines) and dNK (preference for pyrimidines), only ATP1 exhibited weak to moderate activity, and the other five analogues even lower activity, or not at all (Table 4).

The foregoing also apparently correlates with the excellent donor properties of UTP, with both human[19,48] and *B. subtilis*[7] dGK, and with human dCK ([8] and references cited). By contrast, UTP is a poorer donor than ATP for TK1, TK2[8] and dNK,[2] which are in group (a) in Table 4. One apparent exception is dCK, which prefers UTP independently of acceptor,[8] but exhibits restricted donor activity with dAdo (group (a)), and clearly suggests that donor and acceptor activities are mutually interrelated.

Differences Between Highly Purified Natural and Cloned Enzymes

The five enzymes employed in this study were all cloned, and it is of interest, in this context, to compare their activities with their natural counterparts previously reported.[16] For native dCK, K_m values, and Hill coefficients (in brackets), for ATP, ATP1 and ATP2 (with dCyd as acceptor) are $47 \pm 3\,\mu M$[49] ($h = 0.7$), $25 \pm 5\,\mu M$ ($h = 1$) and $20 \pm 3\,\mu M$ ($h = 1$), respectively, as compared to $7 \pm 1\,\mu M$ ($h = 0.7$), $11.0 \pm 1.5\,\mu M$ ($h = 0.8$–1) and $7.4 \pm 0.3\,\mu M$ ($h = 1$) for the recombinant enzyme (Table 5). For native TK2, the equivalent values for ATP and ATP2 (with dThd as acceptor) were $10.0 \pm 1.5\,\mu M$[50] ($h = 0.4$) and $50 \pm 6\,\mu M$ ($h = 1$), respectively, as compared to $2\,\mu M$ ($h = 1$) and $36\,\mu M$ ($h = 0.7$) for the recombinant enzyme (Table 5). For TK1, with ATP2 as donor and dThd as acceptor, K_m values and Hill coefficients were similar. Cloned dNK exhibited the same acceptor properties as the native enzyme, but donor properties were not described.[22,51] For cloned dGK[18,52] no such

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results have been reported. One conceivable interpretation of the foregoing differences between native and cloned enzymes is the existence of post-translational modifications of the former, e.g., it has been suggested that phosphorylation of dCK[53–55] and TK1[56] may regulate their activity in situ.

Structural Aspects: Mode(s) of Binding of Donors and Acceptors

Crystal structures of the complex of dNK with dCyd, and of dGK with ATP have been recently reported,[40] and the catalytic sites identified. In the dNK/dCyd complex, dCyd is bound, as expected, in the nucleoside binding site. By contrast, and unpredictably, ATP is bound by dGK (but in the absence of a nucleoside acceptor) in a direction opposite to the phosphate donor site, with the base at the deoxyribonucleoside site, as expected for a bisubstrate analogue feedback inhibitor. Furthermore, modeling of dCK, based on dGK (which shares high sequence similarity to dCK, with $\approx 45\%$ identity), revealed no major differences in the side-chains linking the substrate pocket, but with Ala100 in dCK replaced by Ser114 in dGK only 4 Å from a purine substrate, in accord with the good activity of dCK for purine substrates, but unable to account for the good substrate properties of dCyd (and Cyd).

Our previous results,[16] and the present findings, add a totally new element to the foregoing, which must be taken into account in studies of the modes of binding of donors and acceptors by nucleoside kinases in general, particularly if we bear in mind the present results demonstrating *interdependence of binding of donors and acceptors*. We venture to predict that similar considerations probably apply to other kinase systems.

It is, perhaps, relevant to underline the difficulties inherent in qualitative modeling of protein/ligand interactions from crystal structures alone, admirably illustrated by the crystal structures of RNase A in complexes with its two most potent inhibitors,[14] the 2′- and 3′-phosphates of 5′-phosphoadenosine (referred to above), which proved to be unpredictable, and totally at variance, with earlier crystal structures of RNase/inhibitor complexes. It is now obvious that the crystal structures of dCK, and/or dGK, complexed with one of our 2′ (3′)-triphosphate analogues, are essential for further clarification of mode(s) of binding of donors and acceptors by the enzymes embraced in the present investigation. Also required are modeling studies which take into account the simultaneous binding of donors and acceptors, now under way in our laboratories.

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