An NMR conformational study of the complexes of $^{13}C/^{2}H$ double-labelled 2′-deoxynucleosides and deoxycytidine kinase (dCK)

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The structures of the bound $^{13}C/^{2}H$ double-labelled [(2′*R*/*S*,5′*R*/*S*)-$^{2}H_2$-1′,2′,3′,4′,5′-$^{13}C_5$]-2′-deoxyadenosine (dAdo) and the corresponding 2′-deoxycytidine (dCyd) moieties in the complexes with human recombinant deoxycytidine kinase (dCK) have been characterized for the first time by solution NMR spectroscopy. Transferred dipole–dipole (DD) cross-correlated relaxation (CCR) and transferred NOE (TRNOE) experiments have been employed to show that the ligands (*i.e.* dCyd and dAdo) adopt a South-type sugar conformation when bound to dCK. The bound South-type 2′-deoxynucleosides are likely to be in a "near transition state", such that they can be transformed into their 5′-monophosphates by transesterification from the phosphate-donor, ATP. The chemical integrity of the product, 2′-deoxynucleoside 5′-monophosphate, has been unequivocally proven by ^{31}P–^{1}H correlation spectroscopy. It has also been observed that the aromatic–H1′ NOE crosspeaks are the same sign for the bound acceptor and products, the 5′-monophosphate of dAdo or dCyd and ADP, thereby showing that the acceptor and the products have comparable correlation times, and suggesting that they are all bound to the dCK in a ternary complex. Evidence is presented suggesting two binding sites for dAdo compared to dCyd, which has only one binding site on dCK. Our present understanding of the "near transition state" structure of the ligands in the dCK reaction complex may help the design of new nucleoside transition state therapeutics.

Introduction

Deoxycytidine kinase (dCK) is a cytosolic enzyme that plays a key role in the activation of therapeutic nucleoside analogues by their 5′-phosphorylation.[1,2] Nucleoside 5′-triphosphates, *e.g.* adenosine and uridine 5′-triphosphates (ATP and UTP), act as phosphate donors and a broad range of nucleosides serve as acceptors, including anticancer drugs, such as arabinosylcytosine, 2′,2′-difluorodeoxycytidine and 2-chlorodeoxyadenosine, as well as antiviral compounds, *e.g.* 2′,3′-dideoxycytidine.[2] The kinetic properties of the enzyme are complex and earlier steady state and pre-steady state kinetic studies have demonstrated the existence of several conformational states of dCK and the conformational changes occurring upon binding of phosphate donors and acceptors.[1–4] There are also two other cellular enzymes, thymidine kinase 2 (TK2) and deoxyguanosine kinase (dGK), localized to the mitochondria, that show amino acid sequence and functional similarities to dCK and these three enzymes form an enzyme family.[2,5] Other important members of this enzyme family are the nucleoside kinases of the herpes viruses, which possess several regions of sequence similar to those of dCK, TK2 and dGK. The 3D structure of HSV-1 thymidine kinase has been determined and it is the only deoxynucleoside kinase structure known to date.[6,7]

The herpes kinases have broad substrate specificities for nucleoside analogues in that they phosphorylate acyclic and unnatural stereoisomeric forms.[6,7] Some hypermodified racemic compounds such as β-DL-(±)-2′,3′-dideoxy-3′-thiacytidine (BCH-189; 3TC) are also phosphorylated by nucleoside kinases.[8,9] Interestingly, both isomers of 3TC show anti-HIV activity and they are both taken up and converted by dCK to the corresponding monophosphates by cultured cells.[8,9] The component with the unnatural β-L-(−)- configuration shows better antiviral effect and lower cytotoxicity than its β-D-(+)-counterpart, and 3TC is now one of the most used anti-HIV drugs. A number of L-nucleosides have been synthesized and studied for their substrate specificities against dCK.[8–10] A comprehensive study of the stereoisomeric specificities of the cellular deoxynucleoside kinase with four stereoisomers of natural deoxynucleoside was recently published.[11]

One of the structural elements which is, however, common in all of these nucleoside analogues is that they all consist of unsymmetrically substituted saturated five-membered (pentose) sugar rings, and the nature of their exocyclic substituents limits the flexibility of the otherwise flexible pentose or thiapentose system and leads to their preferred puckering modes.[12a] Various X-ray and NMR studies[12b] show that the conformation of the pentose sugar moiety in nucleosides can be adequately described by a two-state equilibrium [N $\rightleftharpoons$ S] between the North-type (N, C2′-*exo*-C3′-*endo*, $0 \le P \le 36°$, $\Psi_m = 36 \pm 3°$) and the South-type (S, C3′-*exo*-C2′-*endo*, $144 \le P \le 190°$, $\Psi_m = 36 \pm 3°$) pseudorotamers, where P is the phase angle of pseudorotation and Ψ_m is the puckering amplitude. The N $\rightleftharpoons$ S equilibrium is energetically controlled by the interplay of the steric and stereoelectronic *gauche* and anomeric contributions[12] of various sugar substituents.

All the above studies on the pseudorotational transitions of the pentose sugar in a nucleoside are based on the conformational analysis of the pure "stand-alone" compound in solution—not in the form of any complex. We argue that it will be a considerable advantage for the design of potentially important antiviral/antitumour sugar-modified nucleosides to

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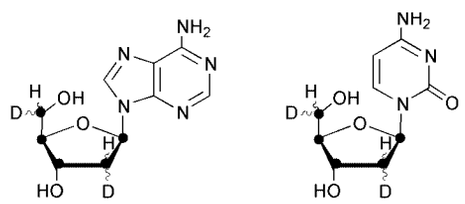
know which pseudorotational state is preferred by dCK as the transition state in a complex between a 2′-deoxynucleoside and dCK for the transesterification reaction by ATP.

We herein present our NMR evidence from transferred dipole–dipole (DD) cross-correlated relaxation (CCR)[13] and transferred NOE (TRNOE)[14–17] experiments. It shows that the flexible pentose sugar moieties of both dAdo and dCyd in the bound state with dCK adopt a South-type conformation. This is, most probably, a "near transition state" conformation involved in the phosphate transfer reaction with ATP in the *in vitro* synthesis of 2′-deoxyribonucleoside 5′-monophosphate.

Recently the quantitative measurement of DD cross-correlation relaxation has been proposed by Griesinger *et al.*[13a,b] based on the relaxation of multiple quantum coherences effected by DD cross-correlation.[13] This new approach has been applied for conformational studies of protein[13b] and RNA.[13a] The ratio of the crosspeak intensities in the two experiments (cross and reference experiments) bears a simple relationship to the cross-correlated rate, ($\Gamma^{c}_{C_iH_iC_jH_j}$), and hence to the angle subtended by the internuclear vectors,[13a,b] C_iH_i and C_jH_j. For the ribofuranosyl moiety in a nucleoside derivative, the determination of the sugar pucker amplitude is based[13a] on the ratio of the two cross-correlated rates: $\Gamma^{c}_{C_1H_1C_2H_2}/\Gamma^{c}_{C_3H_3C_4H_4}$.

For 2′-deoxynucleoside derivatives, this method could not be applied in a straightforward manner because of possible interference of DD cross-correlation between the $C_{2'}H_{2'}$ and $C_{2'}H_{2''}$ vectors at C2′. The only remedy we envisioned was to use chemospecific deuterium labelling at the C2′ position. Earlier, we had shown[18] that the chemospecific incorporation of deuterium at C2′ and C5′ in the ^{13}C-labelled sugar moiety eliminates the geminal H2′–H2″ and H5′–H5″ couplings, facilitating the homo and hetero *J*-coupling analyses, and thereby helping to eliminate the cross-correlation effect of DD(^{13}C–^{1}H) from the ^{13}C relaxation rate of the methylene protons.

We herein report on a conformational study of complexes of [(2′*R*/*S*,5′*R*/*S*)-$^{2}H_2$-1′,2′,3′,4′,5′-$^{13}C_5$]-2′-deoxyadenosine (**1**), and the corresponding 2′-deoxycytidine (**2**) derivatives [bearing diastereomeric proton and deuteron in 1:1 ratio at C5′, and 15% (*R*):85% (*S*) at C2′] with dCK by means of transferred dipole–dipole cross-correlated relaxation and transferred NOE experiments.



The results documented in this paper are the first report on the conformational analysis of 2′-deoxynucleosides bound in the form of a ligand–protein complex.

Experimental

(I) Synthesis of double ^{13}C/^{2}H labelled nucleosides

The syntheses of ^{13}C/^{2}H double-labelled nucleosides have been performed using published procedures.[19] For NMR measurements, a 5 mM concentration of nucleosides in 0.6 mL D_2O was used with different concentrations of dCK.

(II) Enzyme preparation and purification

The dCK mutant (S8F) has been constructed (unpublished data), cloned and expressed using the pET-9d bacterial vector system.[20] Expression of the dCK coding DNA was induced by the addition of IPTG† and growth was continued for 4 h at 37 °C. Cells were harvested by centrifugation at 5000 rpm for 10 min at 4 °C, resuspended and lysed by freeze-thawing and sonication (3 × 1 min) on ice in 20 mM Tris-HCl (pH 7.9), 0.5 M NaCl and 1 mM PMSF.‡ The lysate was then centrifuged at 45000 rpm for 1 h at 4 °C and dCK was then purified by metal chelate affinity chromatography. After unbound proteins were washed away, dCK was eluted with 0.5 M imidazole in 20 mM Tris-HCl (pH 7.9), 0.5 M NaCl and 1 mM PMSF. The purity of the enzyme was determined using SDS-polyacrylamide gel electrophoresis. After purification the elution buffer was changed to 10 mM potassium phosphate buffer (pH 7.3) using PD-10 columns Sephadex® G-25 (Pharmacia Biotech). Protease cleavage was performed by using Thrombin (Pharmacia) as described earlier.[20] Cleavage was monitored by SDS-polyacrylamide gel electrophoresis.

(III) Gel filtration chromatography

Gel filtration chromatography was performed using fast protein liquid chromatography on a Superdex® 200 column with the Pharmacia Monitor UV-II (Pharmacia Biotech) operating at 280 nm and flow rate 0.4 ml min^{-1}. The column was equilibrated and eluted with a buffer containing 10 mM potassium phosphate buffer (pH 7.3). BSA§ (M_r 66 000) and carbonic anhydrase (M_r 29 000) were used as molecular weight markers. Protein fractions corresponding to 60 kDa were collected and concentrated with the centrifugal filter device, Ultrafree®-15 (Millipore).

To prepare the protein sample for NMR experiments the 10 mM potassium phosphate buffer in H_2O was replaced by the same buffer in D_2O, where the final D_2O concentration was 90%. The sample was concentrated to 0.6 ml and a protein concentration of 60 mg per ml by the centrifugal filter device.

(IV) Enzyme assay

dCK activity was routinely followed by a radiochemical assay procedure, as has been described earlier using 2′-[5-^{3}H]dCyd.[20] Assays were performed in 50 mM Tris-HCl (pH 7.6), 100 mM KCl, 5 mM $MgCl_2$, 5 mM ATP, 2 mM DTT,¶ 0.5 mg ml^{-1} BSA, 50 ng of pure dCK and 25 μM [^{3}H]dCyd.

(V) NMR experiments

The NMR experiments were carried out on a Bruker DRX spectrometer at a magnetic field strength of 14.1 T, operating at 600.13 MHz for ^{1}H, 150.92 MHz for ^{13}C and 92.12 MHz for ^{2}H, and at a magnetic field strength of 11.7 T operating at 500.03 MHz for ^{1}H, 125.74 MHz for ^{13}C and 76.76 MHz for ^{2}H. Both spectrometers were equipped with a Bruker digital lock and with a switching ^{2}H lock-^{2}H pulse device.

The 600.13 MHz spectrometer was equipped with an inverse detection quadro-resonance probehead with triple axis gradients for ^{1}H, ^{13}C, ^{31}P and ^{15}N (QXI). Hard ^{1}H pulses were applied with 29 kHz. ^{13}C hard pulses were applied with 19.2 kHz. ^{13}C decoupling was performed using GARP[21a] with 3.84 kHz field strength. For the 90° and 180° ^{2}H pulses the probe power after the switching block was 6.4 W, which corresponds to a 2.08 kHz applied field. ^{2}H decoupling utilized a WALTZ16[21b] sequence using a 588 Hz field.

The 500.03 MHz spectrometer was equipped with triple-resonance probehead for ^{1}H, ^{13}C and ^{2}H (TXO) and TXN. For the TXO probe: ^{1}H and ^{13}C pulses were applied with 24 kHz and 40 kHz fields, respectively. ^{13}C decoupling was performed using GARP with a 4.17 kHz field strength. For the 90° and

† Isopropyl β-D-thiogalactopyranoside.
‡ Phenylmethylsulfonyl fluoride.
§ Bovine albumin.
¶ Dithiothreitol.

180° ^{2}H pulses, the probehead power after the switching block was 43.0 W, which corresponds to an 11.4 kHz applied field. ^{2}H decoupling utilized a WALTZ16 sequence with a 1.3 kHz field (0.6 W). For the TXN probe: ^{1}H and ^{13}C pulses were applied with 24 kHz and 40 kHz fields, respectively. ^{13}C decoupling was performed using GARP with a 4.17 kHz field strength. For the 90° and 180° ^{2}H pulses, the probehead power after the switching block was 43.0 W, which corresponds to an 11.4 kHz applied field. ^{2}H decoupling utilized a WALTZ16 sequence using a 1.3 kHz field (0.6 W). To avoid the spinning artefacts, all spectra were measured on non-spinning samples.

(i) NOE-transfer experiments. 2D TRNOESY experiments of weakly bound complexes were performed as recently published[16] at a magnetic field strength of 11.7 T. To eliminate the signal from the protein a spinlock pulse of 2500 Hz was applied.[16] The theoretical analysis of 2D TRNOESY was limited to the initial build-up rates, which is valid for short mixing times.[14,15] In the present work the interproton distances were determined from the time dependence of the relative volumes of crosspeaks at several mixing times: 35, 70, 100 and 150 ms. Assessment of the linear NOE build-up curves at different mixing times (data not shown) allows us to assume the validity of the two-spin approximation under our experimental conditions[15] up to the mixing time, $\tau_m = 100$ ms.

With fast exchange between the bound and free states of the ligand with the protein and assuming the validity of the two spin approximation, the intensity of the crosspeak in the TRNOE experiment is given by eqn. (1), where σ_{ij}^b and σ_{ij}^f are the

$$a_{ij}(\tau_m) \cong -(p_b\sigma_{ij}^b + p_f\sigma_{ij}^f)\tau_m \quad (1)$$

cross relaxation rates between spin i and j for the bound and free states, τ_m is the mixing time, and p_b and p_f are the fractions of the bound and free states, respectively. The cross relaxation rate is defined through spectral density function, $[J_n(\omega)]$, as eqn. (2),[14,15,22] where the spectral density functions take the form of eqn. (3), in which τ_c is the rotational correlation time

$$\sigma_{ij} = (1/10)(\gamma^4\hbar^2/r_{ij}^6)[6J_2(\omega) - J_0(\omega)] \quad (2)$$

$$J_n(\omega) = \tau_c/[1 + (n\omega\tau_c)^2] \quad (3)$$

assuming the isotropic tumbling, ω is the angular Larmor frequency for the proton, γ is the gyromagnetic ratio of the proton and $n = 0, 1, 2$. Eqns. (2) and (3) show that at very short ($\omega\tau_c \ll 1$, for free ligand) and very long ($\omega\tau_c \gg 1$, bound ligand to protein) correlation times the cross relaxation rate is proportional to τ_c. Moreover, even at $p_b \sim 0.05$ for proteins as large as 60 kD with $\tau_c > 80$ ns,[15] the contribution of the free conformation to the crosspeak is small due to its very short τ_c (≤1 ns) and eqn. (1) can be simplified to eqn. (4).

$$a_{ij}(\tau_m) \cong -(p_b\sigma_{ij}^b)\tau_m \quad (4)$$

The cross-relaxation rates can then be used to calculate the interproton distances (r_{ij}) by eqn. (5), under a two-spin

$$\frac{\sigma_{ij}}{\sigma_{kl}} = \left(\frac{r_{kl}}{r_{ij}}\right)^6 \quad (5)$$

approximation and the assumption of a single correlation time of the ligand–protein bound complex using a calibration distance of $H_{1'}$–$H_{2'}$ (2.9 ± 0.2 Å[17c,23]).

(ii) Determination of DD cross-correlation rate. Cross-correlated relaxation (CCR) is the dominant process of the ligand in the state bound to protein, compared with the free state[13] due to its linear dependence with the correlation time (τ_c) [see eqn. (6)] in a straight analogy to the TRNOE experiment for weakly bound complex.[14–16] The fast exchange between the bound and the free states enables us to observe the effect of CCR at the resonances of the free ligand.[13] Assuming isotropic overall tumbling, the cross correlated rate is given by eqn. (6),[13a] where $Y_{20}(\theta_{ij}) = (3\cos^2\theta_{ij} - 1)/2$ and $J_a(0) = 2S^2\tau_c/5$,

$$\Gamma^c_{C_iH_iC_jH_j} = \left(\frac{\mu_o\hbar}{2\pi}\right)\frac{\gamma_C^2\gamma_H^2}{r^3_{C_iH_i}r^3_{C_jH_j}}\,Y_{20}(\theta_{ij})J_a(0) \quad (6)$$

S^2 is the generalized order parameter, τ_c is the overall correlation time and θ_{ij} is the angle between two vectors C_iH_i and C_jH_j. The general approach proposed for ribonucleoside[13a] is that the sign of ratio $\Gamma^c_{C_1H_1C_2H_2}$ to $\Gamma^c_{C_3H_3C_4H_4}$ can discriminate between C2′-*endo* and C3′-*endo* conformations.

The DD cross correlation rates, $\Gamma^c_{C_iH_iC_jH_j}$, were measured at a magnetic field strength of 11.7 T using the pulse sequence *quantitative*-Γ-HCCH proposed recently by Griesinger *et al.*,[13a] the only difference being that before ^{13}C chemical shift evolution in t_1 the WALTZ16 modulation on deuterium was used to decouple ^{13}C from deuterium. Two types of spectrum were obtained from a cross and a reference experiment. In a cross experiment the evolving period of two coupling constants, $J_{C_iH_i}$ and $J_{C_jH_j}$, $\Delta = 1/2J_{CH}$, was set up to zero. In a reference experiment $\Delta = 3.36$ ms was applied, which corresponds to $J_{CH} = 148$ Hz. This value is an average between the coupling constants of the sugar moiety: $J_{C_1H_1} \approx 170$ Hz, $J_{C_3H_3} \approx J_{C_4H_4} \approx 140$ Hz and $J_{C_2H_2} \approx 120$ Hz. To obtain the cross relaxation rate, $\Gamma^c_{C_iH_iC_jH_j}$, the ratio of volumes of cross (I_{cross}) to reference (I_{ref}), experiments has been used, [eqn. (7)].[13a]

$$\frac{I_{cross}}{I_{ref}} = \tanh(\Gamma^c_{C_iH_iC_jH_j}\tau_m) \quad (7)$$

The mixing time, τ_m, for the evolution of the double/zero quantum coherence was set to 25 ms to refocus ^{13}C–^{13}C coupling constants.

The data sets were recorded as 4 K × 96 real matrix with 64 scans for each t_1 value and a spectral width of 10 ppm in F2 and 160 ppm in F1 with the carrier for ^{1}H, ^{13}C and ^{2}H at 4.8, 82.84 and 3.25 ppm, respectively. In all cases the recycle delay used was 2.0 s.

(iii) ^{31}P–^{1}H correlation experiments. A standard HSQC type experiment has been performed to obtain inverse proton–phosphorus correlation at a magnetic field strength of 14.1 T. The data sets were recorded as 2 K × 256 real matrix with 64 scans for each t_1 value and a spectral width of 10 ppm in F2 and 30 ppm in F1 with the carrier for ^{1}H, ^{31}P at 4.8 and 0 ppm, respectively. In all cases the recycle delay used was 2.0 s. The 1D ^{31}P spectrum has been measured with proton decoupling.

Results

The dCK enzyme is a dimer composed of two identical 30 kDa subunits. To prepare a protein sample at 1 mM concentration it was necessary to stabilize the protein during the preparation to prevent aggregation and inactivation. Previous work (unpublished data) has shown that a single mutation in position 8 (S8F) in the N-terminal end of dCK leads to an increase in the stability of the protein. This mutation has only minor effects on the enzyme's kinetic behaviour. Expression and purification of the recombinant S8F dCK were done using the pET-9d vector and the *Escherichia coli* strain BL21 (DE3) p Lys S as described earlier.[20] A test for enzyme stability was performed daily by removing 5 µl of protein sample from the NMR tube. No evidence was found to indicate any significant decrease in the specific activity of dCK, even after several months of storage in the NMR tube.

To perform the cross-correlated relaxation (CCR) and transferred NOE (TRNOE) experiments with samples of

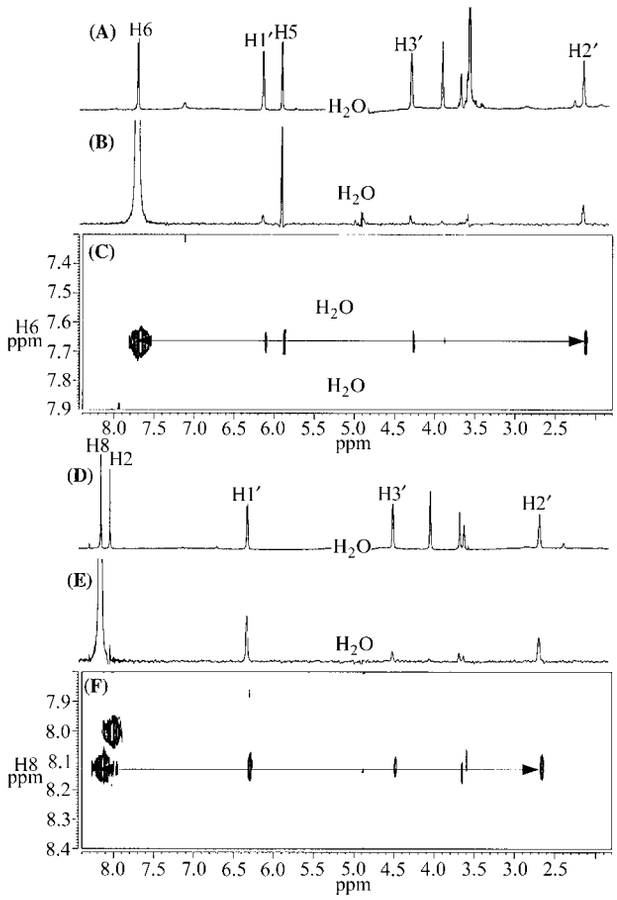


Fig. 1 The expanded H6/8–H1′/H2′/H3′ region in the TRNOESY spectra of dAdo + dCK [Panel (F)] in 10 mM potassium phosphate buffer, and dCyd + dCK [Panel (C)] in 10 mM Tris-HCl buffer. The cross-sections through the H6/H8 aromatic proton in the F2 dimension are shown for dAdo + dCK [Panel (E)] and dCyd + dCK [Panel (B)]. They show that the crosspeaks of the nucleoside in dAdo + dCK and dCyd + dCK complexes have positive signs, like the corresponding diagonal peaks for H6/H8. ^{1}H spectra, together with the assignment of H6/H8, H1′, H2′ and H3′ protons of the deoxynucleosides, are presented for dCyd + dCK [Panel (A)] and dAdo + dCK [Panel (D)].

ligand–protein complexes under the same experimental and environmental conditions, we have used a mixture of 2′-deoxynucleoside consisting of 30% double ^{13}C/^{2}H labelled deoxynucleoside for dAdo and dCyd.

(I) Detection of the weakly-bound complexes of ^{13}C/^{2}H double-labelled nucleosides with dCK

The TRNOE experiments were performed with a solution consisting of dCyd or dAdo and dCK at the natural isotope abundance. Whereas negative NOE signals were detected for the free nucleoside in solution (data not shown), an extensive set of positive NOE signals was detected when the nucleoside was mixed with dCK (Fig. 1C for dCyd and Fig. 1F for dAdo). The detection of the positive crosspeaks in 2D TRNOE spectra [14–17] on the resonance of the free ligand (see the cross-section through the H8/H6 crosspeak in the F2 dimension, Fig. 1B for dCyd and Fig. 1E for dAdo) allows us to conclude that both 2′-deoxynucleosides, dCyd and dAdo, are in fast exchange between the free and the weakly-bound states with dCK, which most probably represents a "near transition state" model for the transesterification reaction with ATP (see below).

To establish the conformation of the bound-nucleosides in dCK, the sugar moieties in dAdo and dCyd were ^{13}C/^{2}H double-labelled in order to measure the CCR rates [eqn. (6), Experimental section], enabling us to assess different endocyclic torsion angles. It is noteworthy that the replacement of H2″ and H5′(H5″) by ^{2}H has eliminated the relaxation due to the strong cross-correlation between two geminally coupled ^{1}H2′–^{1}H2″ and ^{1}H5′–^{1}H5″ protons, thereby facilitating the measurement of the CCR rates in the sugar moiety. Two complementary spectra (cross and reference, see Experimental section for definition) [13a,b] for each ^{13}C/^{2}H double-labelled dAdo and dCyd (5 mM) with dCK (0.1 mM) at 0 °C are shown in Figs. 2 and 3, respectively, showing the CCR crosspeaks between C1′H1′ and C2′H2′. These crosspeaks are observed in the cross-section through resonances of H1′ and ^{13}C2′ for complexes of either dAdo or dCyd with dCK. It is noteworthy that the crosspeaks found in the above CCR cross experiment are absent for free dAdo and dCyd (data not shown) because of their shorter correlation times in the free state [eqn. (6)]. After scaling the intensity of the crosspeak in the corresponding reference experiments by 50 times (because a 1 : 50 ratio of

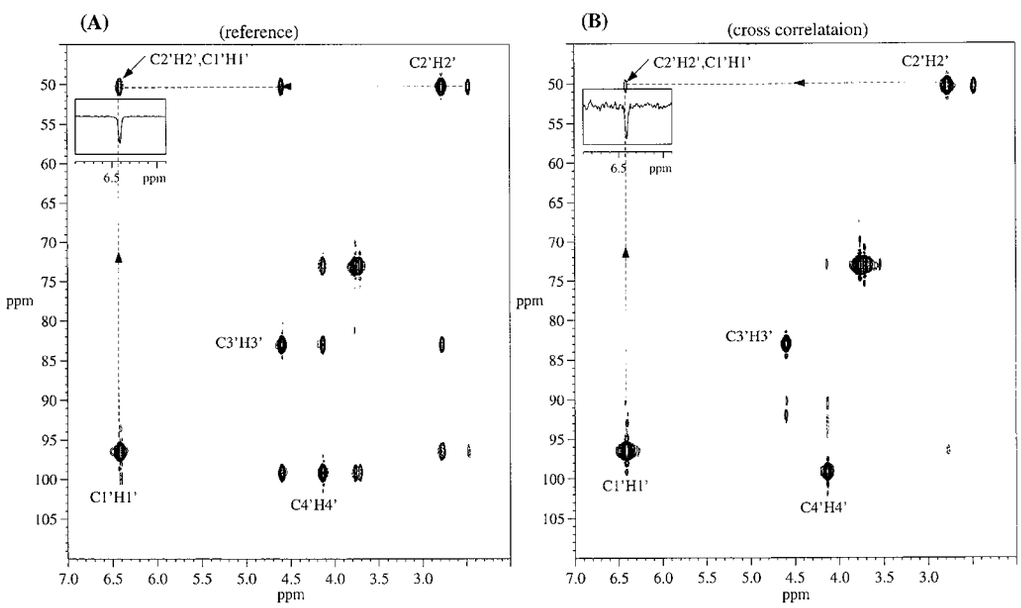


Fig. 2 The CCR reference experiments [Panel (A)] and CCR cross experiments [Panel (B)] for dAdo + dCK (1 : 50) in 10 mM potassium phosphate buffer at 273 K. dAdo was ^{13}C/^{2}H double-labelled in the sugar moiety. The arrow shows the crosspeaks (inset), between the two $C_{1'}H_{1'}$ and $C_{2'}H_{2'}$ vectors. It is noteworthy that the sign of the cross correlated crosspeaks C1′H1′,C2′H2′ in the reference experiment [Panel (A)] and the cross correlated experiment [Panel (B)] is the same.

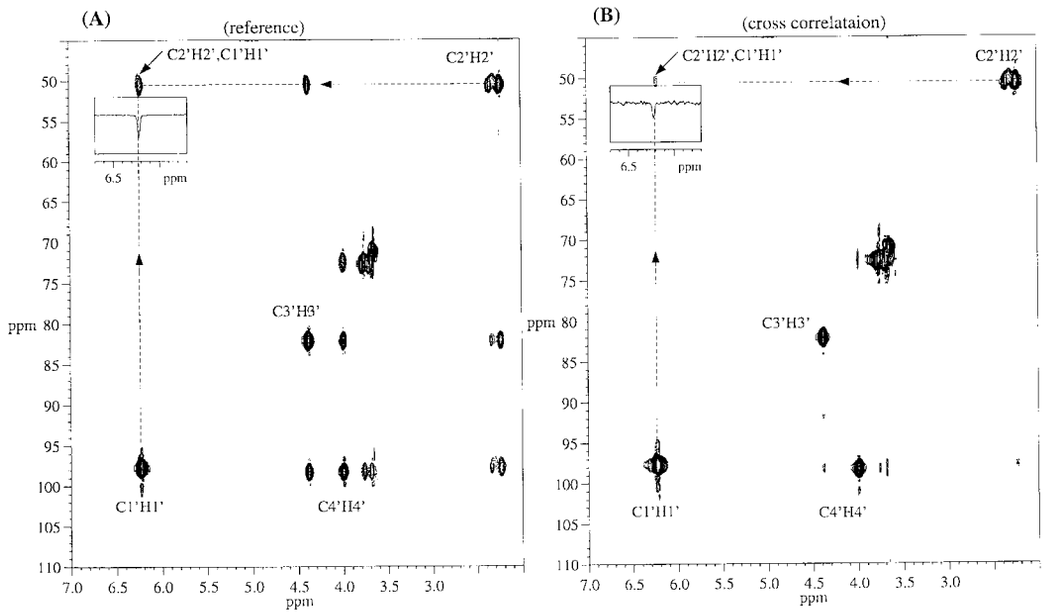


Fig. 3 The CCR reference experiments [Panel (A)] and CCR cross experiments [Panel (B)] for dCyd + dCK (1 : 50) in 10 mM Tris-HCl buffer at 273 K. dCyd was $^{13}C/^{2}H$ double-labelled in the sugar moiety. The arrow shows the crosspeaks (inset) between two $C_{1'}H_{1'}$ and $C_{2'}H_{2'}$ vectors. It is noteworthy that the sign of the cross correlated crosspeaks C1′H1′,C2′H2′ in the reference experiment [Panel (A)] and the cross correlated experiment [Panel (B)] is the same.

the protein to ligand was used in the NMR experiment) for both 2′-deoxynucleosides, the ratios of I_{cross}/I_{ref} are found to be close to 1. This is due to the long correlation time of the 2′-deoxynucleoside–dCK complex. Note that the correlation time (τ_c) is ~90 ns[15] at 4 °C for a protein of similar molecular weight to dCK (60 kDa), thereby making the quantitative analysis of the data difficult; it has not been attempted in the present work. Note, also, that eqn. (7) can be optimally applied for the complex with the overall τ_c between 1–10 ns.[13e] This range of τ_c has been verified for the CCR experiment by using 2′-deoxynucleoside in viscous media (a d_6-ethylene glycol–water mixture) to increase the correlation time (unpublished data).

(II) The conformation of the sugar moiety of dAdo and dCyd weakly-bound to dCK

The qualitative analyses of the CCR experimental data for both dAdo and dCyd bound to dCK show that the ratio I_{cross}/I_{ref} for C1′H1′–C2′H2′ is positive (Figs. 2 and 3). On the other hand, the cross-correlated peaks for C3′H3′–C4′H4′ are very weak (Figs. 2 and 3), and have opposite signs to the corresponding crosspeak in the reference experiments, and the ratio of I_{cross}/I_{ref} is close to zero or negative. Based only on the signs of $\Gamma^{c}_{C_1H_1C_2H_2}$ and $\Gamma^{c}_{C_3H_3C_4H_4}$,[13a] it can be concluded that the sugar moiety of both dAdo and dCyd prefers to adopt a South-type conformation in the bound state with dCK.

Qualitative analysis of the data obtained from the TRNOE spectra shows that in a mixture of dCyd–dCK (Fig. 4A, B), the intensities of the H1′–H6/H3′/H4′ crosspeaks (detected through the cross-section of H1′ chemical shift) are comparable or less intense than the intensity of the H1′–H2′ crosspeak (note that the H1′–H2′ distance is found to be constant at 2.9 ± 0.2 Å,[17c] independent of the sugar conformation).

A different relative aromatic–H1′ crosspeak intensity was, however, observed for dAdo in the dAdo–dCK complex (Fig. 4C and 4D) compared to that of dCyd in the dCyd–dCK complex. The H1′–H8 crosspeak is much more intense than that of H1′–H2′, qualitatively suggesting that the conformation of the aglycone around the glycosyl bond for dAdo in the bound state is most probably *syn* compared to the *anti* conformation found for the bound dCyd (see below for quantitative analysis).

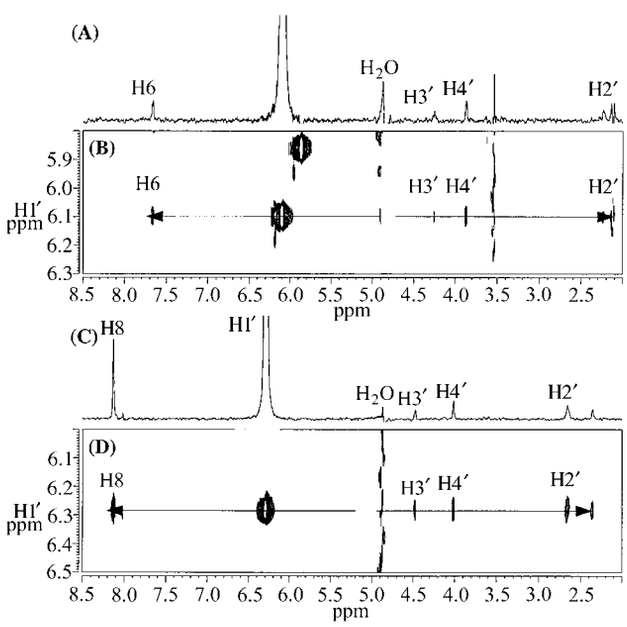


Fig. 4 The expanded H1′–H6/H8–H2′/H3′/H4′ TRNOESY spectra of dCyd + dCK [Panel (B)] in 10 mM Tris-HCl buffer and dAdo + dCK [Panel (D)] in 10 mM potassium phosphate buffer. The cross-sections through the H1′ in the F2 dimension are shown for dCyd + dCK [Panel (A)] and dAdo + dCK [Panel (C)]. They show that the crosspeaks of the nucleoside in the dAdo + dCK and dCyd + dCK complexes have positive signs, like the corresponding diagonal peaks for H1′. The assignments of H6/H8, H1′, H2′ and H3′ protons of dCyd and dAdo are also shown.

The data obtained by the CCR and TRNOE experiments prompted us to perform a quantitative analysis of the TRNOE data to determine both the endocylic and glycosyl torsions. The χ torsion[23,24] can be estimated from a knowledge of the intra-nucleotide distances between the base H6/H8 and the sugar H1′, H2′ and H3′ protons. It is known that amongst these distances the H6/H8–H1′ distance depends only on χ, while other distances depend both on χ and P (phase angle of pseudorotation[24]). It has been shown[23] that the H6/H8–H1′

Table 1 The cross relaxation rates, σ_{ij} (s^{-1}), and experimental distances, r_{ij} (Å), obtained from TRNOE experiments for dAdo and dCyd weakly-bound to dCK

Ligand	Data type	2′–1′	2′–3′	2′–4′	2′–H6	4′–3′	4′–1′	1′–3′	H6–3′	H6–1′	H5–H6
dCyd	r_{ij}	3.0[a]	2.4	—	2.5	3.3	3.0	4.4	3.4	3.3	2.5
	σ_{ij}[b]	1.4 ± 0.5	4.9 ± 1.5	—	4.4 ± 1.5	0.9 ± 0.4	1.7 ± 0.5	0.4 ± 0.3	1.9 ± 0.6	2.0 ± 0.6	12.7 ± 3.0
dAdo	r_{ij}[a]	3.0[a]	2.4	3.3	2.7	2.8	3.1	3.4	3.2	2.5	—
	r_{ij}[c]	3.0	2.4	4.0		3.0		4.0			
	σ_{ij}[b]	5.0 ± 1.0	17.3 ± 2.1	3.3 ± 0.4	9.9 ± 0.9	7.7 ± 0.7	4.3 ± 0.2	2.5 ± 0.2	3.2 ± 0.3	13.8 ± 0.39	—

[a] The distance H1′–H2′ is used as a reference in the two proton approximation. [b] σ is the cross relaxation rate between protons *i* and *j*. [c] The distances which do not show dependence on the conformation of the sugar moiety.

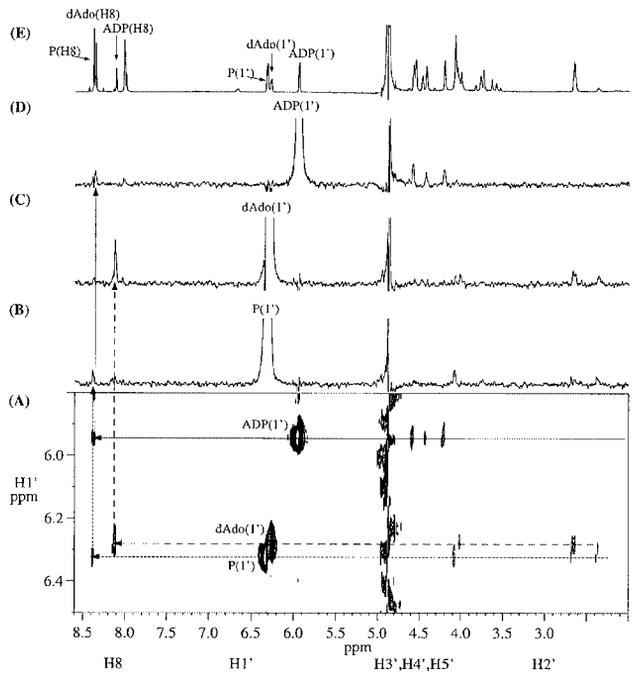


Fig. 5 The expanded H1′–H6/H8/H1′/H3′/H4′/H2′ TRNOESY spectrum [Panels (A)] of the dAdo + dCK–ADP mixture after 24 h. Three products are clearly detected: dAdo [Panel (C)], marked by dAdo(1′); its 5′-phosphorylated product [Panel (B)], marked by P(1′); and the leaving ADP [Panel (D)], marked by ADP(1′). It is evident from these data that all three substances have the same positive sign for both the H8–H1′ NOE crosspeaks and the corresponding H1′ diagonal peaks, suggesting they all involve in a similar interaction with dCK at 273 K.

is ~2.2–2.6 Å for the *syn* and >3.4–3.8 Å for the *anti* conformation. The H6/H8–H2′ varies between 2.0–3.0 Å for the *anti* and 3.0–4.5 Å for the *syn* conformations. Moreover, it is known[23] that the H1′–H4′ distance in the sugar moiety is one of the most sensitive markers (the H2″–H4′ distance could not be used because of C2″-deuterium in the sugar moiety of the nucleoside) for the subtle variation of the sugar conformation.

Interproton distances were determined from the mixing-time dependence of the relative volumes of the NOE cross-peaks obtained from the 2D TRNOESY experiments[14–17] (see Experimental section). The results of the calculated and theoretically expected interproton distances are listed in Table 1. For the dCyd–dCK complex, both qualitative evaluation of the CCR data and quantitative calculation of the interproton distances (H2′–H6) indicate that the sugar moiety adopts a South-type conformation. The distance $r_{H1'-H6} \geq 3.0$ Å $\geq r_{H2'-H6}$ from the TRNOESY experiment suggests that the aglycone adopts an *anti* type conformation.[23] Furthermore, consideration of the H1′–H4′ distance ($r_{H1'-H4'} \geq 3.0$ Å)[23] within the South-type conformational space for the sugar moiety in the dCK–nucleoside complex reflects the fact that *P* is limited to 140–180°.

A more complex picture has, however, been observed for the dAdo–dCK complex. On the one hand, the H8–H1′ distance, $r_{H1'-H8} \sim 2.5$ Å,[23] indicates that the aglycone adopts a *syn* type conformation,[23] and on the other hand our CCR experimental data strongly suggests that the sugar moiety in the bound state adopts the South-type conformation. This *syn*-South conformation should give a H2′–H8 distance much larger than 3 Å (more like 4–5 Å), which is not the case. Indeed it is 2.7 Å, which corresponds more to an *anti*-South type conformation.[23] The question of whether the glycosyl bond is *syn*-South or *anti*-South (based on the distances between H1′–H8 and H2′–H8) can be resolved by assuming at least two binding sites for dAdo at the acceptor as well as at the donor (ATP) binding sites. The experimental evidence supporting this idea has been

obtained from the TRNOE experiment (Fig. 5A), which shows that the signs of all three NOE crosspeaks between the H8–H1′ protons arising from dAdo, its 5′-phosphate and adenosine 5′-diphosphate (ADP), with respect to the corresponding H1′ diagonal peaks, are positive (see Fig. 5B–D). That they all have very similar correlation times suggests that they are all weakly bound to dCK in a similar manner. Thus there appears to be more than one binding site for dAdo on dCK, in contrast to dCyd binding, and in both binding modes dAdo takes up a South-type conformation. In earlier ATP binding studies[17] it was found that ATP adopts a North-type or 1′-*exo* sugar pucker with an *anti* type conformation.

Additional experimental support for the proposal that dAdo utilizes both its own as well as the presumed ATP site comes from a comparison of the absolute values of the observable cross-relaxation rates (Table 1). This shows that the cross-relaxation rate for dAdo is 2–3 times greater than the corresponding rates for dCyd under identical experimental conditions. Assuming that the correlation times for the dCK complexes with dAdo and dCyd are the same, we can conclude that the number of dAdo molecules bound to dCK is higher than that for dCyd, even though dCyd is a more efficient substrate for the enzyme. This suggests that the high K_m for dAdo, compared to dCyd, as the phosphate acceptor could be due to dAdo competing with ATP for binding at the donor site on dCK. However, there are no clear cut enzyme kinetic results supporting this conclusion, but the kinetic behaviour is very complex, with positive and negative cooperativity reported in the dCK reaction with its substrates.[1–4] Further studies are in progress to resolve this issue.

(III) Detection of the phosphorylation product in the deoxynucleoside–dCK–ATP complex

The formation of a weakly bound complex between dAdo or dCyd and dCK is presumably a prerequisite in order to proceed to the 5′-phosphorylation step in the presence of a phosphate donor such as ATP. To demonstrate the appearance of the product of the actual transesterification reaction, *i.e.* the phosphorylation of the 2′-deoxynucleoside–dCK complex with ATP, 1D ^{31}P, ^{13}C and ^{1}H and 2D ^{31}P–^{1}H correlation experiments were performed.

(A) dAdo + dCK + ATP mixture. The 1D ^{31}P spectra of a mixture of dAdo–dCK (50 : 1 molar ratio) (Fig. 6A) shows only one ^{31}P resonance from the phosphate buffer itself. The reaction mixture of dAdo–dCK (50 : 1 molar ratio, see Experimental section) containing ATP (2.5 mM) after 30 min shows five resonances in the upfield region between −2 and −20 ppm: three belong to ATP and two belong to the departing by-product ADP, formed as a result of the phosphate transesterification reaction. After 30 min, the ^{31}P resonance for α^{ATP} at −9.7 ppm and two new appearing resonances of ADP at −5.6 and −9.5 ppm could be clearly seen (Fig. 6B). Two other resonances of ATP, at −5.2 (γ^{ATP}) and −17.9 ppm (β^{ATP}) could not be observed in this experiment due to severe line broadening. After 12 h, the reaction was found to be complete (Fig. 6D) and only two resonances from ADP at −5.6 and −9.5 ppm are observed. The assignment of these resonances has been based on a ^{31}P–^{1}H correlation experiment (Fig. 7). The resonance at −9.5 ppm shows a crosspeak with H5′/5″ protons, showing that it is α^{ADP}, but the resonance at −5.6 ppm does not show a crosspeak with any proton, suggesting that this is β^{ADP}.

The assignment of the new ^{31}P signal at 4.8 ppm [Fig. 6B, C and D; labelled p(5′)] is based on the following observation. As mentioned above, the ATP used in these experiments had natural ^{13}C abundance, but in the mixture of dCK–dAdo, the deoxynucleoside component consisted of 30% ^{13}C labelled sugar moiety, which leads one to expect that the H5′(or H5″)–

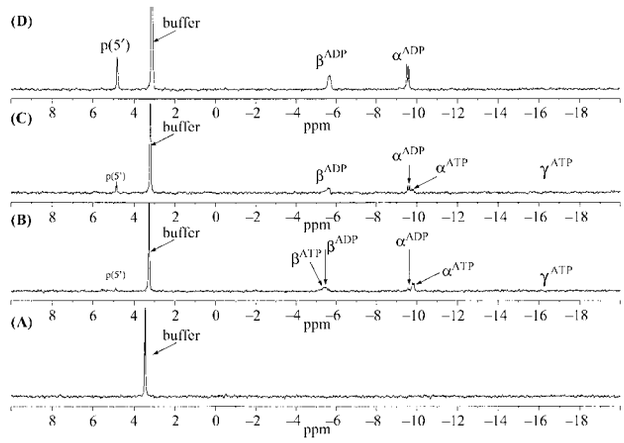


Fig. 6 ^{31}P Spectra of (A) dAdo–dCK (1 : 50) in 10 mM potassium phosphate buffer at 273 K, (B) dAdo–dCK after addition of ATP, (C) dAdo–dCK + ATP after 2 h and (D) dAdo–dCK + ATP when the reaction is complete (12 h). The product of 5′-phosphorylation is labelled p(5′). The signal from the phosphate buffer is indicated by "buffer". The ^{31}P resonances of ATP and ADP are indicated by arrows with the corresponding assignment.

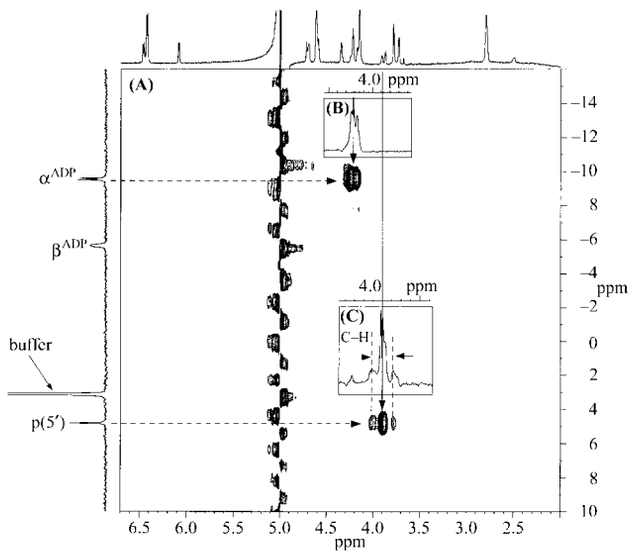


Fig. 7 [Panel (A)] The ^{1}H–^{31}P correlation spectra of the dAdo–dCK + ATP mixture when the reaction is complete (after 12 h) in 10 mM potassium phosphate buffer at 273 K. The product of 5′-phosphorylation is labelled p(5′). The cross-sections through the crosspeaks are presented showing the singlet for ^{31}P(α^{ADP}) of ADP [inset (B)] and triplet for 5′-phosphorylated dAdo [inset (C)]. 1D ^{1}H (on the top of the panel) and ^{31}P (on the left side of the panel) spectra are also shown.

^{31}P crosspeak for ADP or ATP will be present in the ^{1}H–^{31}P spectrum as a singlet; but the corresponding crosspeak in the 5′-phosphorylated product will be resolved in the F2 as a triplet. Two of the peaks of the triplet are due to the ^{13}C–^{1}H coupling constant splitting of ~170 Hz, and the middle component of the triplet is due to the ^{12}C–^{1}H part of the product (*i.e.* a singlet superimposed in the middle of the doublet). Indeed, the new phosphorus resonance at 4.8 ppm, which appeared after the addition of the ATP to the dCK–dAdo mixture, shows the crosspeak with the 5′,5″ protons. The projection in the F2 dimension through this crosspeak, (Fig. 7, Panel C), shows a clear triplet with an expected intensity ratio close to 15 : 70 : 15 due to the isotopic mixture composition. This data allowed us to attribute the ^{31}P resonance at 4.8 ppm to 5′-phosphorylated 2′-dAdo.

(B) dCyd + dCK + ATP mixture. In analogy with the dAdo–dCK + ATP reaction, the formation of the 5′-phosphorylation

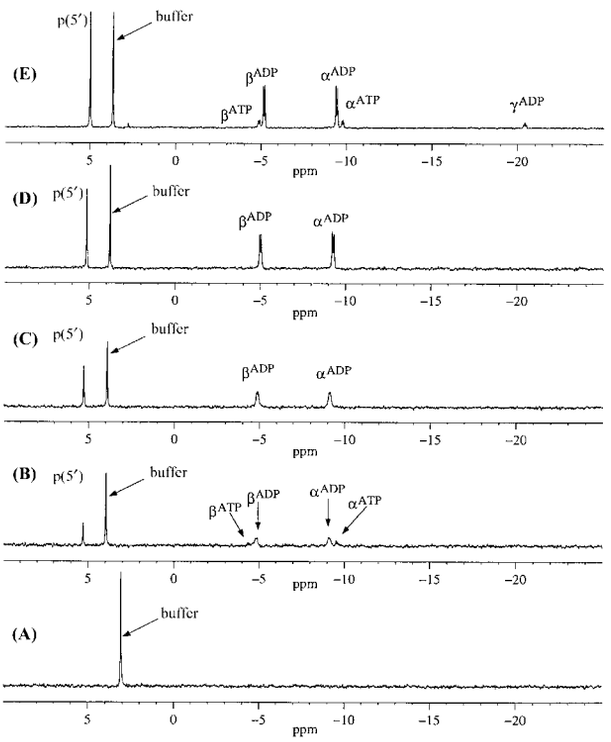


Fig. 8 ^{31}P Spectra of (A) dCyd–dCK (1 : 50) in 10 mM phosphate buffer without DTT at 293 K, number of scans (ns) = 1k; (B) 30 min after addition of ATP to the dCyd–dCK complex at 293 K, ns = 1k; (C) 24 h after addition of ATP to the dCyd–dCK complex at 293 K, ns = 1k; (D) one week after the addition of ATP to the dCyd–dCK complex, at 283 K, ns = 1k and (E) one month at 273 K, ns = 4k. The product of 5′-phosphorylation is labelled p(5′). The signal from the phosphate buffer is indicated by "buffer". The ^{31}P resonances of ATP and ADP are indicated by arrows with the appropriate assignment.

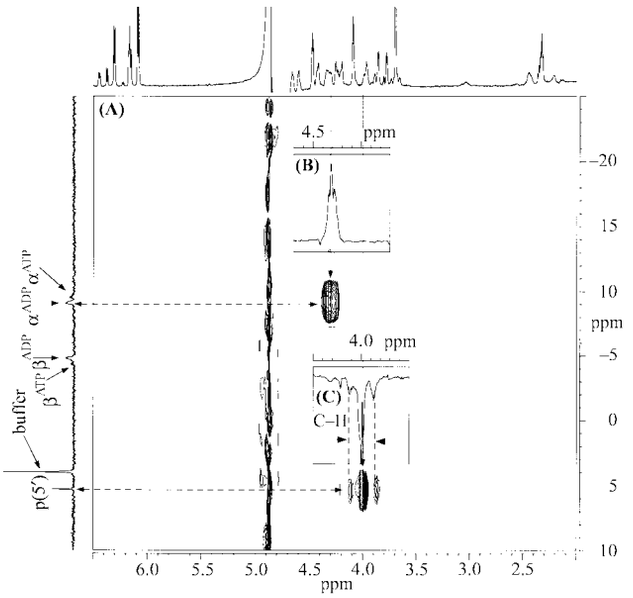


Fig. 9 [Panel (A)] ^{1}H–^{31}P Spectra of dCyd–dCK and dCyd–dCK + ATP when reaction is complete in 10 mM potassium phosphate buffer (without DTT) at 273 K. The product of 5′-phosphorylation is labelled p(5′). The cross-sections through the crosspeaks are presented showing the singlet for ^{31}P(α^{ADP}) [inset (B)] of ADP and triplet for 5′-phosphorylation product of dCyd [inset (C)]. ^{1}H (on the top of the panel) and ^{31}P (on the left side of the panel) spectra are also shown.

product from the dCyd–dCK–ATP complex was evident from the appearance of a new resonance at 5.1 ppm (Fig. 8) but in this case reaction was much faster. The ^{1}H–^{31}P correlation experiment (Fig. 9B) shows crosspeaks at 5.1 ppm (^{31}P) and 4.0 ppm (H5′/H5″). A cross-section through the crosspeak shows a well resolved triplet (Fig. 9D) in the F2 dimension in analogy with the structure of the 5′-phosphorylated dAdo (see Fig. 7C).

It is noteworthy that the appearance of the 5′-phosphorylated product was observed in the above phosphorylation reactions without the addition of $MgCl_2$ to the reaction mixture. The addition of $MgCl_2$ did not qualitatively facilitate the phosphorylation process studied in the NMR time scale.

Conclusion

(1) Based on the TRNOE and CCR experiments, the "near transition state" conformations of the weakly bound complexes between dAdo/dCyd and dCK have been identified and characterized. The TRNOE data shows that the aglycone of dCyd adopts the *anti* conformation and the sugar moiety is locked into the 2′-*endo* conformation in the dCK binding site.

(2) The TRNOE data for dAdo bound to dCK is consistent with the dAdo binding in two different sites on dCK, presumably at the dAdo site and the ATP binding site. The two bound dAdo molecules adopt two different combinations of aglycone and sugar conformations: one is a *syn*-South conformation and the other is an *anti*-South.

(3) The fact that in our NMR experiment on the dCK complex, no Mg^{2+} ion seems to be required as cofactor for the transesterification reaction supports the idea that ATP in the dCK active site is capable of adopting an active phosphate conformation necessary for activity as a phosphate donor. Clearly, this is only possible if the 5′-phosphoryl moiety of ATP is in close electrostatic contact with some specific residues in dCK in the proximity of the ATP binding site, facilitating the scission of the terminal phosphate ester bond of ATP. It is still possible that there is some Mg^{2+} bound to dCK, but it should be substoichiometric compared to ATP, dAdo or dCyd. Work is in progress to clarify this issue.

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