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Human Deoxycytidine Kinase as a Deoxyribonucleoside Phosphorylase

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Human deoxycytidine kinase (dCK) is a key enzyme in the 5′-phosphorylation of purine and pyrimidine deoxynucleosides with deoxycytidine as the most efficient substrate. The ability of dCK to degrade 2′-deoxyribonucleosides to free nucleobases and 2-deoxy-α-D-ribofuranose-1-phosphate was demonstrated by ^{1}H–^{31}P correlation spectroscopy and by isotope enzyme kinetic methods. The reaction depended on inorganic phosphate, and dCK showed maximum cleavage activity between pH 7 and pH 8. In this pH range, [HPO_4^{2-}] is the dominant phosphate species, most likely being the phosphate donor. All natural deoxyribonucleosides could be cleaved and the V_{max} of the phosphorylytic reaction compared to the kinase reaction was about 2–10%. The formation of free nucleobases occurred only with reduced dCK, because the reaction was highly dependent on the presence of reducing agents such as dithiotreitol. Thus, recombinant dCK can act as a phosphorylase, similar to the nucleoside phosphorylase family of enzymes. This catalytic activity is important for the design of *in vitro* experiments with dCK, such as crystallization and NMR spectroscopy.

Keywords: deoxycytidine kinase; nucleoside analogs; phosphorylation; phosphorolysis reaction; nucleoside phosphorylase

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] 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 conformational changes occurring upon binding of phosphate donors and acceptors.[1–6] The structures of the bound 2′-deoxyadenosine (dAdo) and 2′-deoxycytidine (dCyd) moieties have been characterized in complex with human recombinant deoxycytidine kinase by solution NMR spectroscopy using $^{13}C/^{2}H$ double-labeled 2′(*R*/*S*), 5′(*R*/*S*)-$^{2}H_2$-1′,2′,3′,4′,5′-$^{13}C_5$-2′-deoxy-β-D-nucleosides (Figure 1).[4] During this study it was crucial to find optimum conditions for the NMR experiments that allow the observation of the weakly bound complex of dCK and dCyd (or dAdo). We noticed that in some cases, even in the absence of ATP as phosphate donor, a degradation of the nucleosides (dCyd, dAdo and dThd) occurred. To avoid the possibility of contamination by bacterial enzymes responsible for this kind of degradation, recombinant human dCK was purified extensively by fast protein liquid chromatography (FPLC).

This study is the first report on the ability of dCK to degrade 2′-deoxyribonucleosides to free nucleobases and 2-deoxy-α-D-ribofuranose-1-phosphate (2-deoxyribofuranose-1-phosphate: P(1)-deoxyribose), as demonstrated by ^{1}H–^{31}P correlation spectroscopy and ^{3}H-labeling methods. The goal of the present study was to clarify the structure of the new products and the condition for their formation. The new reaction was studied with regard to several factors, such as concentration of the enzyme, 2′-deoxyribonucleosides, temperature and pH. The reaction required a phosphate-containing buffer. The pH optimum of the activity suggested that HPO_4^{2-} was one of the substrates.

Abbreviations used: dCK, deoxycytidine kinase; HSQC, heteronuclear single quantum spectroscopy; PNPase, purine nucleoside phosphorylase; UDPase, uridine phosphorylase; TDPase, thymidine phosphorylase.

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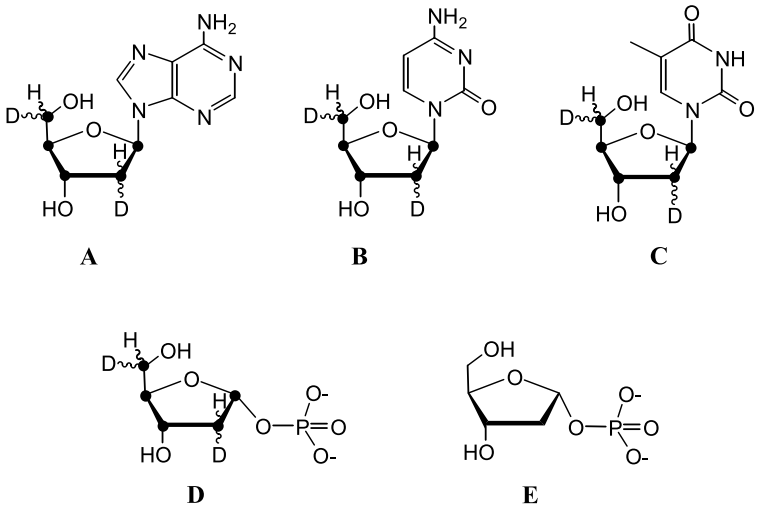

Figure 1. The structures of $^{13}C/^{2}H$ double-labeled nucleosides (A–C) and the product (D) as well as the reference non-labelled compound (E). A, 2′(*R*/*S*), 5′(*R*/*S*)-$^{2}H_2$-1′,2′,3′,4′,5′-$^{13}C_5$-2′-deoxyadenosine (dAdo); B, 2′(*R*/*S*), 5′(*R*/*S*)-$^{2}H_2$-1′,2′,3′,4′, 5′-$^{13}C_5$-2′-deoxycytidine (dCyd); C, 2′(*R*/*S*), 5′(*R*/*S*)-$^{2}H_2$-1′,2′,3′,4′, 5′-$^{13}C_5$-thymidine (dThd); D, 2′(*R*/*S*), 5′(*R*/*S*)-$^{2}H_2$-1′,2′,3′,4′, 5′-$^{13}C_5$-2-deoxy-α-D-ribofuranose-1-phospate [P(1)-deoxyribose product]; E, reference native compound, 2-deoxy-α-D-ribofuarnose-1-phospate. In compounds A–D, the percentage of the isotope enrichment is as follows: the H2′(*S*) and H2′(*R*) mixture is ca 85 : 15 atom % ^{2}H, and all sugar carbon atoms are ^{13}C-labeled (98 at.% ^{13}C) in 30% of the nucleosides, the H5′(*R*)/H5′(*S*) mixture contains ca 50 at.% ^{2}H.[4]

The apparent K_m and V_{max} values for all natural deoxyribonucleosides (dCyd, dThd, dAdo and dGuo) were determined.

Results

In our earlier study,[4] surprisingly, after adding the substrate (dCyd) at a concentration of 5 mM to the sample of 0.1 mM protein, we observed the appearance of a new set of $^{1}H–^{13}C$ cross-peaks after three hours at 20 °C in the heteronuclear single quantum spectroscopy (HSQC) NMR spectrum, indicating the formation of a new compound containing a pentose-sugar moiety. In Figure 2(A), a spectrum is presented for the above reaction recorded at 0 °C. In this study $^{13}C/^{2}H$ double-labeled 2′(*R*/*S*), 5′(*R*/*S*)-$^{2}H_2$-1′,2′,3′,4′,5′-$^{13}C_5$-2′-deoxynucleosides were used (Figure 1)[4] to obtain the one-bond $^{1}H–^{13}C$ correlation for substrates without interference from the resonance from the protein. For comparison, Figure 2(B) shows the HSQC spectrum of 5 mM nucleoside substrate immediately after mixing with the same amount of dCK protein.

Our hypothesis was that dCK, during the reaction with dCyd, could cleave the 2′-deoxynucleosides to the free nucleobase and P(1)-deoxyribose (Figure 1D). This hypothesis was supported by the experimental data obtained from ^{1}H and ^{13}C resonance assignments based on the CCR experiments, ^{31}P assignment based on $^{1}H–^{31}P$ correlation experiments and ^{3}H labeled detection of the free nucleobase, as described below.

To exclude bacterial contamination of the samples as a possible reason for degradation of the nucleosides (e.g. bacterial phosphorylases), we have added different antibiotics such as kanamycin, ampicillin and chloramphenicol to the samples at the start of the incubations. Still, the same degradation reaction was observed, a strong indication that only dCK preparation was the reason for the transformation of the nucleosides to free nucleobases and P(1)-deoxyribose as products. This type of product is known to be formed during the phosphorylytic cleavage of 2′-deoxyribonucleosides by nucleoside phosphorylases using inorganic phosphates as a reaction partner (Figure 3).[7]

Preparation of protein

To avoid the possible contamination of dCK by the proteins from *Escherichia coli* BL21 (DE3) pLysS host cells the dCK protein was purified by a two-step purification protocol. Recombinant dCK was purified by metal chelate affinity chromatography using Ni-NTA His Bind® Resin followed by gel-filtration chromatography in 10 mM potassium phosphate buffer. dCK is a dimer with a molecular mass of 60 kDa. The *E. coli* genome encodes three nucleoside phosphorylases; purine nucleoside phosphorylase (PNPase), uridine phosphorylase (UDPase), and thymidine phosphorylase (TDPase).

The subunit sizes calculated from the deduced amino sequences are 27.95 kDa for UDPase, 47.3 kDa for TDPase, and 25.95 kDa for PNPase. The molecular masses of the native enzymes correspond to a homohexamer for UDPase (6× 27.15 kDa), a homodimer for TDPase (2×47.3 kDa), and a homohexamer for PNPase (6×25.95 kDa).[8–10] Using FPLC it should be possible to separate dCK protein from these possible contaminating enzymes.

Potassium phosphate buffer was chosen for the NMR experiments to avoid additional proton signals in the spectra. Moreover, to prepare a highly active and stable protein sample at a concentration of 1 mM, we found that addition of 10 mM DTT throughout the preparation procedure was necessary. A test for enzyme stability was performed daily by removing 5 µl of protein-substrate sample from the NMR tube, and no evidence was found for any

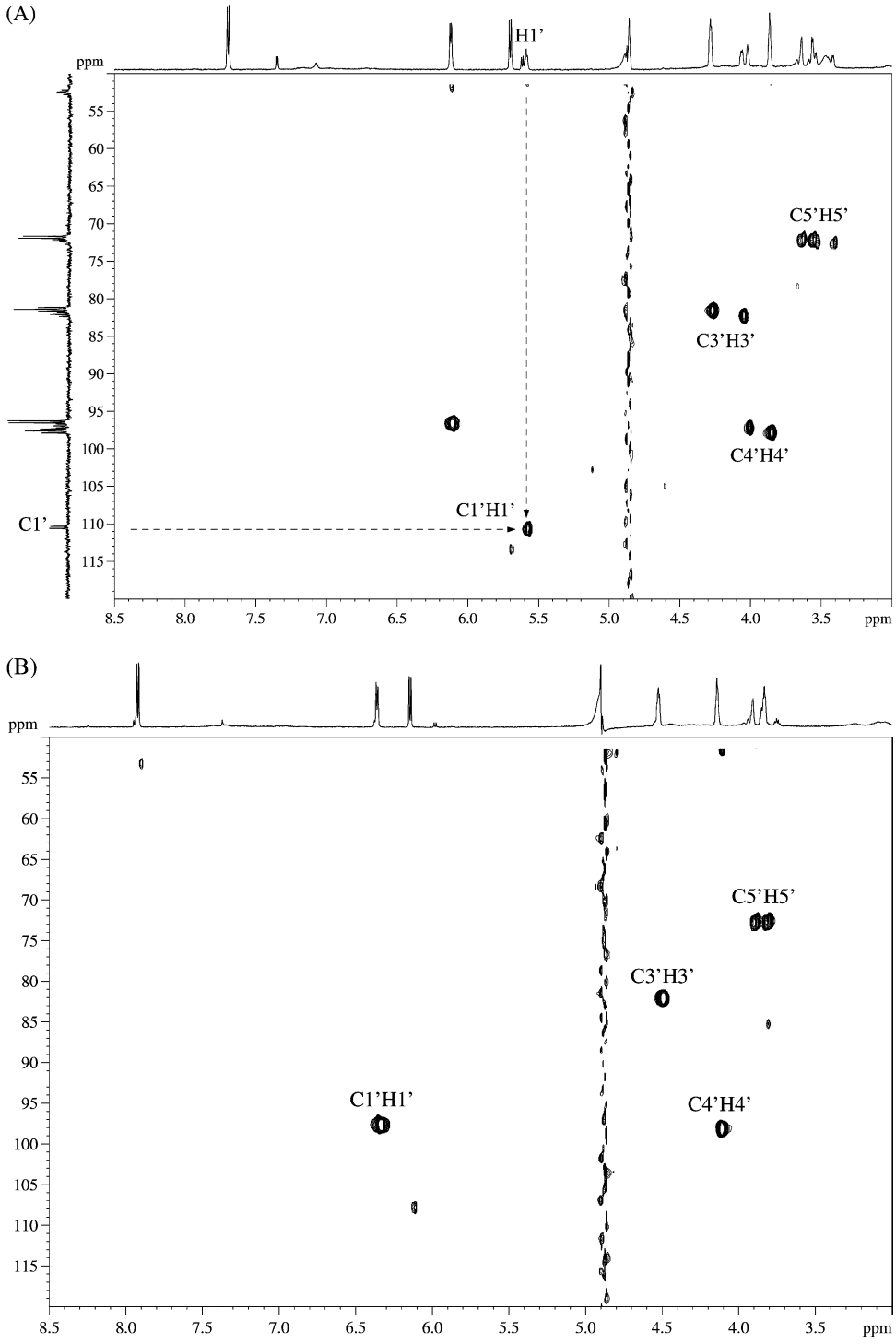

Figure 2. ^{1}H–^{13}C correlation spectra (HSQC) of 0.1 mM dCK, 5 mM dCyd of samples prepared with 10 mM DTT in 10 mM potassium phosphate buffer after five hours of incubation at 20 °C (A) with the NMR data collection performed at 0 °C (in order to minimize degradation), and (B) immediately after addition of dCyd. In (B), the cross-peaks of the sugar moiety of dCyd are marked according to the positions in the sugar (Figure 1). Assignments of the cross-peaks are presented in Figure 4.

significant decrease in the specific activity of dCK even after several months of storage in the NMR tube.

CCR ^{1}H–^{13}C experiments

To assign the ^{13}C and ^{1}H resonances of dCyd as well as those of the new P(1)-deoxyribose product, CCR ^{1}H–^{13}C reference type (see Materials and Methods) experiments have been used. It is known that this experiment[4] allows us to establish both one-bond ^{1}H–^{13}C correlated resonances (in the same way as in HSQC experiments) as well as sequential connectivity between those resonances

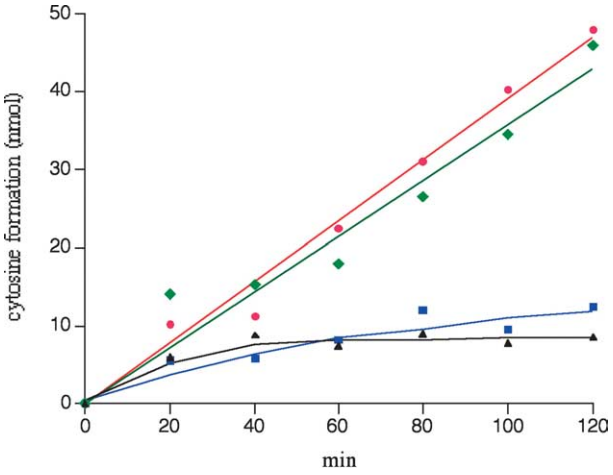

Figure 3. Effects of DTT on the cleavage reaction. The lines and correspond to 1 mM dCK and and correspond to 0.1 mM dCK. The assay was performed in 10 mM potassium phosphate buffer with 5 mM dCyd at room temperature. The formation of cytosine (nmol) in the presence of DTT is shown by and lines, and by and lines in the absence of DTT.

through cross-peaks between the vectors $^{1}H_i$–$^{13}C_i$ and $^{1}H_j$–$^{13}C_j$, if $^{13}C_i$ and $^{13}C_j$ are covalently bound. The CCR reference spectrum of dCyd at 0 °C (Figure 4(A)) and the mixture of dCyd (5 mM) with 0.1 mM dCK after one day of reaction at 0 °C (Figure 4(B)) are presented for comparison. The sequential connectivities starting at the terminal vector H1′C1′ through H2′C2′, H3′C3′, H4′C4′ and H5′C5′ are shown in Figure 4(A) and (B) for dCyd, and in Figure 4(B) for P(1)-deoxyribose. These data lead us to conclude that the new P(1)-deoxyribose product contains a pentose sugar moiety, in which the H1′ and C1′ resonances absorbed at δ 5.4 ppm and ~δ 110 ppm, respectively (Table 1). The ^{13}C and ^{1}H chemical of the new P(1)-deoxyribose product with those of dCyd as well as with those of the reference compound, 2-deoxy-α-D-ribofuranose-1-phosphate, are presented in Table 1. The comparison of Δ_1 and Δ_2 for the ^{13}C and ^{1}H resonances (Table 1) of the new P(1)-deoxyribose product with those of the reference compound, 2-deoxy-α-D-ribofuranose-1-phosphate, indeed shows that their NMR spectroscopic properties are identical within the experimental error, i.e. they are the same compounds.

^{1}H–^{31}P experiments

The structure of the modified products appearing during the incubation of 2′-deoxynucleosides and dCK in the presence of inorganic phosphate was further studied by 1D ^{31}P, ^{1}H and 2D ^{1}H–^{31}P correlation experiments (Figure 5). The 1D ^{31}P spectrum of a mixture of dCyd and dCK (50 : 1 molar ratio) without DTT in 10 mM potassium phosphate buffer showed only one ^{31}P resonance from the phosphate buffer itself (data not shown). The same reaction mixture, after 24 hours with DTT showed a new ^{31}P signal at 2.4 ppm compared with the buffer signal at 3.2 ppm (Figure 5(A)). The assignment of the new signal is based on an approach used by us previously.[4] The double-labeled $^{2}H/^{13}C$-deoxynucleoside component consists of 30% ^{13}C labeled sugar moiety. If incorporation of the inorganic phosphate takes place at some endocyclic sugar carbon centers, then the corresponding methine proton and phosphorus cross-peak in the ^{1}H–^{31}P spectrum of this phosphorylated product will be resolved in the *F*2 dimension as a triplet (Figure 5(B) and (C)). The two outer 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 ^{12}C–^{1}H part of the product (i.e. a singlet superimposition in the middle of the doublet) (Figure 5(C)). Indeed, the new phosphorus resonance at 2.4 ppm, which appeared after the addition of DTT to a mixture of dCK and dCyd showed a ^{31}P cross-peak with the H1 proton (Figure 5(B)). The projection in the *F*2 dimension through this cross-peak (Figure 5(C)) revealed a clear triplet with an expected intensity ratio close to 15 : 70 : 15 due to the isotopic mixture composition, as stated above. These data show unambiguously that the ^{31}P resonance at 2.4 ppm is due to the C1-phosphorylated (P1)-deoxyribofuranose product (Figure 1D).

For comparison with the results presented above, we have performed a control experiment with an NMR sample containing 5 mM dCyd, 0.1 mM dCK (50 : 1 molar ratio), 5 mM ATP in 10 mM potassium phosphate buffer but without DTT after 24 hours of incubation. The ^{1}H–^{31}P correlation experiment of the reaction mixture showed a new downfield signal at 4.5 ppm, which indicated the formation of the dCyd 5′-phosphate (5′-dCMP) product (Figure 6(A)–(C)) in the sample (for the assignment, see Maltseva *et al.*[4]).

To demonstrate the formation of the P(1)-deoxyribose by dCK with dAdo and dThd in presence of ATP in 10 mM potassium phosphate buffer, 1D ^{31}P, ^{1}H and 2D ^{1}H–^{31}P correlation experiments were performed and analyzed in the usual manner after 48 hours of incubation. The results are shown in Supplementary Material (Figures 1S and 2S).

In the 1D ^{31}P spectra (Supplementary Material, Figure 1S(A)), three ^{31}P resonances were found: one from the phosphate buffer itself at 3.2 ppm, the second from the 5′-phosphorylated dAdo (5′-dAMP) at 3.8 ppm, and the third peak at 2.4 ppm belongs to a small amount of the P(1)-deoxyribose, which was confirmed by the cross-peak resolved in the *F*2 dimension as a triplet (Supplementary Material, Figure 1S (B) and (C)) in the ^{1}H–^{31}P spectrum. The NMR property of the 5′-phosphorylated dAdo product, 5′-dAMP, is consistent with the properties of 5′-dCMP, as described earlier.[3]

Similar results were obtained with 5 mM dThd

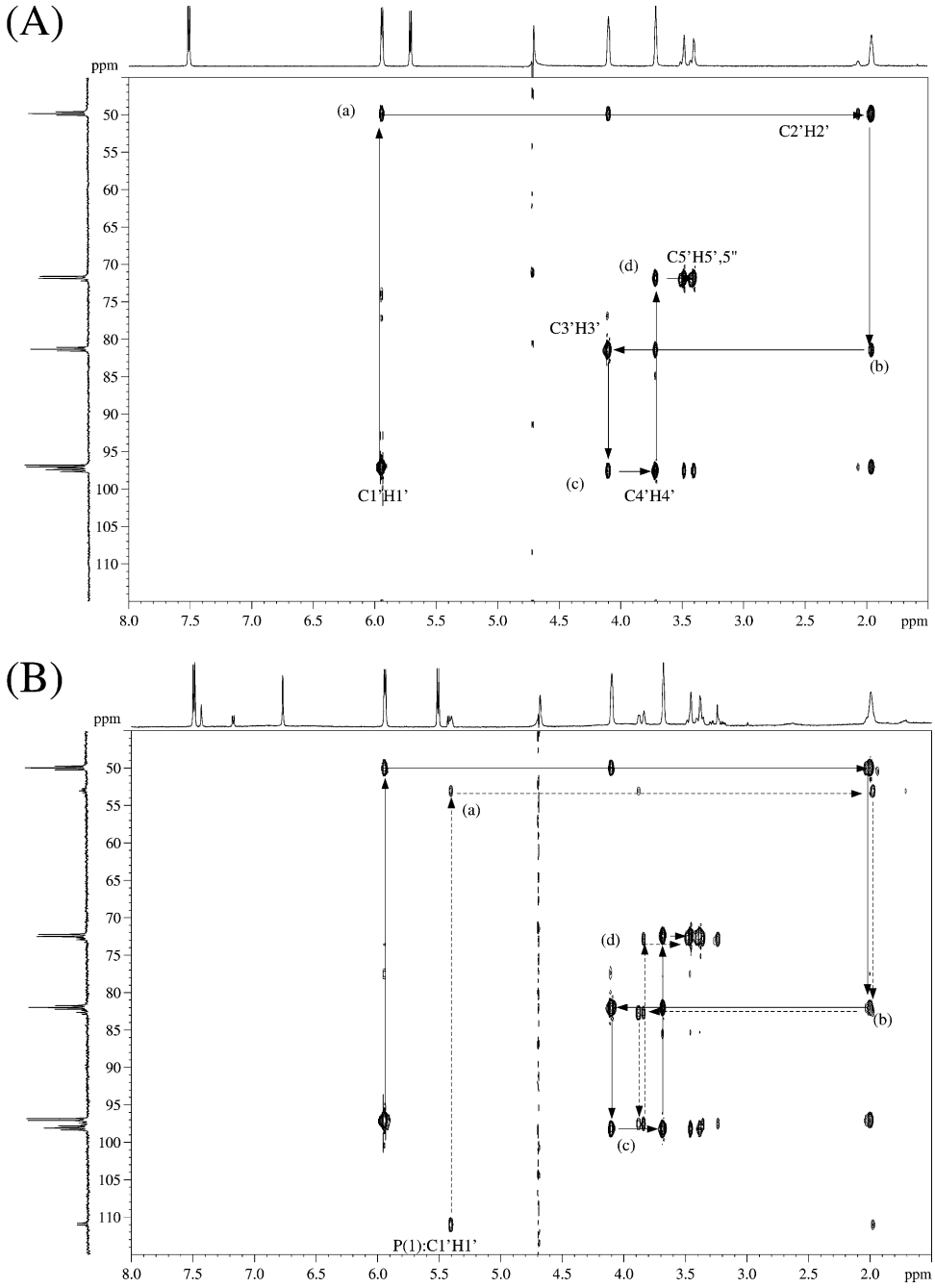

Figure 4. The CCR reference experiments for (A) dCyd and (B) dCyd plus dCK (1 : 50) in 10 mM potassium phosphate buffer with 2 mM DTT at 273 K after one day of reaction. (A) and (B) For dCyd, the sequential connectivities between two vectors, $C_i'H_i'$ and $C_{(i+1)}'H_{(i+1)}'$ (where $i=1, 2, 3, 4$ are the ^{13}C centers in the sugar moiety) are shown by a continuous black line with an arrow starting from the C1′H1′ cross-peak. One-bond ^{13}C and ^{1}H peaks are labeled by the corresponding number of the carbon centers in the sugar moiety. The cross-correlated cross-peaks labeled by (a) for C1′H1′ and C2′H2′ vectors, (b) for C2′H2′ and C3′H3′ vectors, (c) for C3′H3′ and C4′H4′ vectors, and (d) for C4′H4′ and C5′H5′ vectors. (B) Two sets of connectivities are shown, one from the starting material and the second from the product. The first is identical with that presented in (A) for the dCyd sugar moiety. The second is a newly observed connectivity (dotted line) from the P(1) product. This new connectivity is started from the one-bond ^{13}C–^{1}H cross-peak labeled P (1): C1′H1′. Symbols (a), (b), (c), and (d) show corresponding cross-correlated cross-peaks between two vectors, C_iH_i and $C_{(i+1)}H_{(i+1)}$, in the new product P(1)-deoxyribose.

and 0.1 mM dCK in 10 mM potassium phosphate buffer with 10 mM DTT and 5 mM ATP as presented in Supplementary Material, Figure 2S. Three ^{31}P resonances were also observed (Supplementary Material, Figure 2S(A)) and two of them belong to the new products: the 5′-TMP and the P(1)-deoxyribose from the phosphorylase reaction.

These results demonstrated clearly that both the kinase and phosphorylase reactions could indeed take place with dCyd, dAdo and dThd, but with

Table 1. Comparison of the chemical shifts (δ in ppm) of dCyd, the P(1)-deoxyribose product (Figure 1D), observed during reaction, and the reference compound, 2-deoxy-α-D-ribose-1-phosphate (Figure 1E)

^{1}H or ^{13}C resonances (δ in ppm)	dCyd	P(1)-deoxyribose product (D)	Reference compound (E)	Δ_1[a]	Δ_2[b]
H1′	5.94	5.40	5.40	0.54	0.54
H2′[c]	2.00	1.97	1.99[d]	0.03	0.01
H3′	4.10	3.87	3.87	0.23	0.23
H4′	3.68	3.84	3.83	−0.16	−0.15
H5′/5″	3.45/3.38	3.35/3.23	3.36/3.23	–	–
C1′	96.95	110.91	110.73	−13.96	−13.78
C2′	49.93	53.04	53.00	−3.11	−3.07
C3′	81.96	82.64	82.44	−0.68	−0.48
C4′	98.12	97.46	97.22	0.66	0.90
C5′	72.38	72.78	72.89	−0.40	−0.51
^{31}P	–	2.36	2.40	–	–

The numbering system for carbon and proton nuclei of the sugar moiety of P(1)-deoxyribose product as well as reference compound is the same as that used for dCyd, for comparison (see Figure 1).

[a] $\Delta_1 = \delta(\text{dCyd}) - \delta[\text{P(1)-deoxyribose product}]$.

[b] $\Delta_2 = \delta(\text{dCyd}) - \delta$ [reference].

[c] The H2′(*S*) and (*R*) mixture is ca 85 : 15 atom % ^{2}H.

[d] For reference native compound δ(H2″) = 1.73 ppm has been found. In dCyd and the P(1)-deoxyribose product, H2″ protons have been substituted by deuterium.

different efficiencies (Table 2), in the presence of inorganic phosphate, DTT and ATP.

Finally, to analyze if there was a requirement for the phosphate ions, we have exchanged the potassium phosphate buffer for a Tris–HCl buffer (containing DTT) during the preparation of dCK. There was no P(1)-deoxyribofuranose product detected in the samples after 48 hours of incubation of the components described above (data not shown). Addition of ATP to this reaction mixture led to the formation of a 5′-phosphorylated product during one hour.

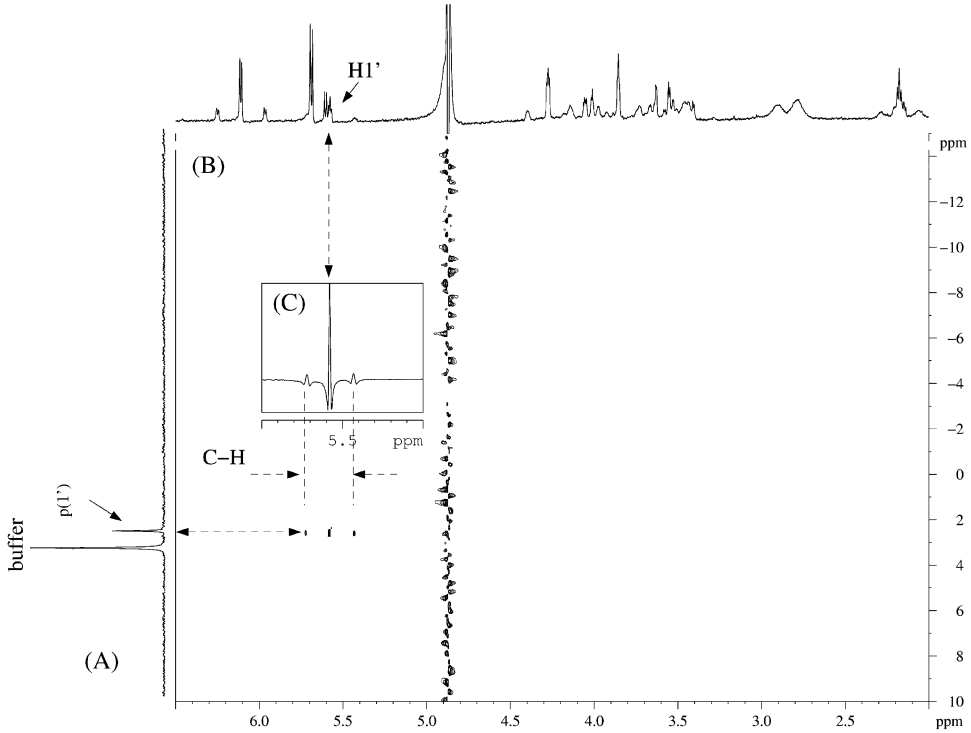

Figure 5. (A) The ^{31}P spectrum of reaction mixture containing 0.1 mM dCK, 5 mM dCyd with 10 mM DTT in 10 mM potassium phosphate buffer at 20 °C after 48 hours. The product of C1′ modification is labeled P(1′). (B) The ^{1}H–^{31}P correlation spectrum of the same sample as in (A), showing the only cross-peaks that can be observed in the P(1)-deoxyribose product spectrum. The cross-section through this cross-peak is presented in (C), demonstrating a triplet. The 1D ^{1}H spectra (at the top of (B)) is shown. The shift of the H1′ proton corresponds to that presented in Figures 2(A) and 4(B) assigned to the newly appearing product.

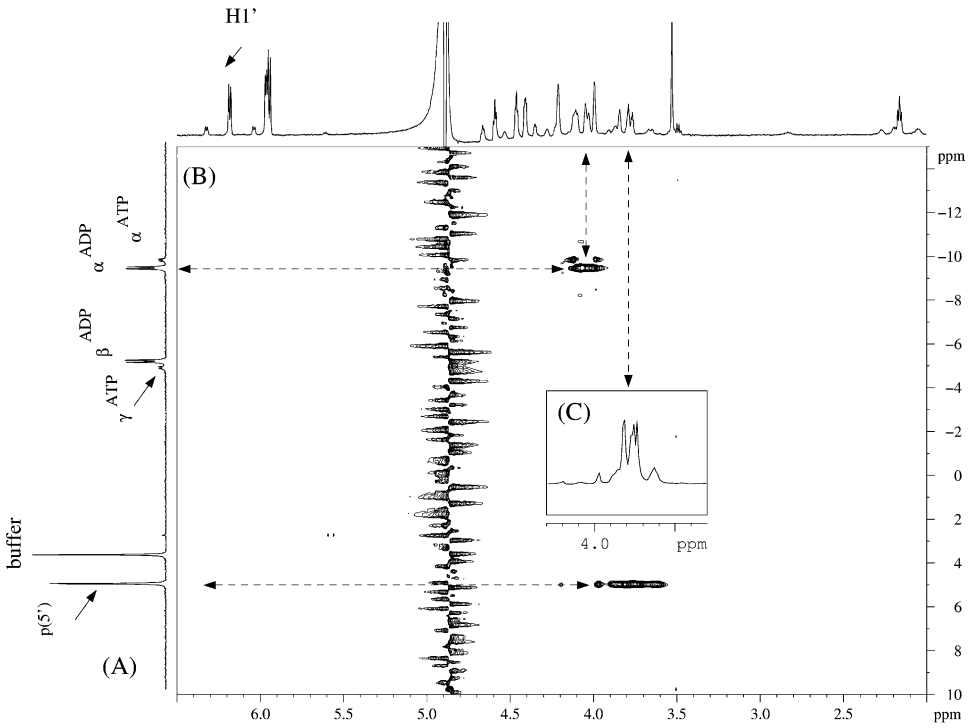

Figure 6. (A) The ^{31}P spectrum of 0.1 mM dCK, 5 mM dCyd, 5 mM ATP in a sample without DTT in 10 mM potassium phosphate buffer at 20 °C after 48 hours. The product of the 5′ modification is labeled P(5′). (B) The ^{1}H–^{31}P correlation spectrum of the same sample. The cross-section through this cross-peak is presented as a triplet for 5′ phosphorylated dCyd (inset in (C)). The 1D ^{1}H spectrum (at the top of (B)) is shown.

These results show that the inorganic phosphate participated in the cleavage reaction catalyzed by human deoxycytidine kinase enzyme.

Effects of DTT and inorganic phosphate on the cleavage reaction

During the NMR study it was observed that the formation of P(1)-deoxyribose occurred preferentially with reduced dCK, because the reaction required the presence of the reducing agent, DTT. Figure 3 shows the formation of cytosine from dCyd with 10 mM DTT and without DTT using 1 mM and 0.1 mM dCK, respectively. The assay was performed in 10 mM potassium phosphate buffer with 5 mM dCyd at room temperature. The samples were analyzed using the radiochemical assay and product formation was confirmed by HPLC analysis. The presence of DTT was important, since the amount of cytosine formed after two hours without DTT was about 10 nmol, whereas with DTT it was about 50 nmol.

An initial test of the effects of different concentrations of phosphate buffer showed that the formation of P(1)-deoxyribose decreased drastically if the concentration of buffer was reduced fivefold, i.e. below 2 mM (data not shown).

pH optimum for the reaction

The two potassium phosphate salts K_2HPO_4 and KH_2PO_4 were used to make the buffers, and the effect of pH on the phosphorylase activity was determined. The formation of cytosine at different pH values as well as the dCyd kinase activity in the same buffers is shown in Figure 7. The optimal activities for both types of reactions were between pH 7 and pH 8 but the enzyme had a broad pH

Table 2. Kinetic parameters for the phosphorylase (A) and phosphorylation (B) activities of human recombinant dCK

Nucleoside		K_m (mM)	V_{max} (nmol/min^{-1} mg^{-1})	K_{cat} (s^1)	K_{cat}/K_m (M^{-1} s^{-1})
dCyd	A	13.2±1.7	0.88±0,07	0.044×10^{-2}	3.3×10^{-2}
	B	0.0015	68±10	0.034	2.310^4
dThd	A	8.1±0.7	0.48±0.02	0.024×10^{-2}	3.0×10^{-2}
	B	0.27±0.04[a]	25±3.7[a]	0.14	5.2×10^{2}
dAdo	A	1.1±0.3	0.02±0.01	0.011×10^{-3}	1.1×10^{-2}
	B	0.16±0.02	1280±192	0.64	0.4×10^{4}
dGuo	A	3.9±2.7	0.03±0.08	0.015×10^{-3}	0.4×10^{-2}
	B	0.12±0.02	360±54	0.18	0.2×10^{4}

[a] Phosphorylation data are from an earlier study.[22]

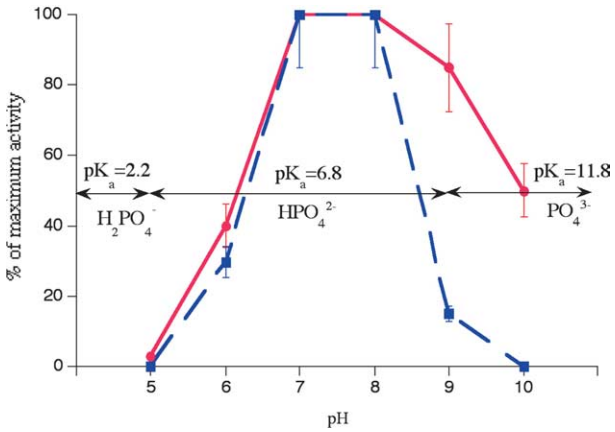

Figure 7. Effects of pH on the formation of cytosine and dCMP. The results are expressed as the percentage of maximum dCK phosphorylated (—●—) and cleaved (—■—). The 100% formed values are 68 nmol min^{-1} mg^{-1} for dCMP and 6.8 nmol min^{-1} mg^{-1} for cytosine.

optimum for the phosphorylation reaction (pH 6–10). The phosphorylase reaction showed a narrower optimum, i.e. between pH 5 to 8 and low activity at higher pH values. Within the pH range 5–9 the dominant ion is HPO_4^{2-} (pK_a 6.8) and this is most likely the active component in the cleavage reaction.[11,12]

The phosphorylase activity with purine and pyrimidine deoxyribonucleosides

The capacity of dCK to degrade different purine and pyrimidine deoxyribonucleosides, e.g. Thd, dAdo and dGuo, was determined and compared to that of dCyd. All reactions were performed using radiolabeled nucleosides where the formation of nucleobases was detected by thin-layer chromatography (TLC) and HPLC analysis.

Kinetic parameters for the phosphorylase and phosphorylation activity of human recombinant dCK are shown in Table 2. The phosphorylase activity was determined after two hours and the phosphorylation data (V_{max}, K_m, K_{cat}, and hence K_{cat}/K_m) were determined by monitoring formation of deoxyribonucleoside-5′-monophosphates. The efficiency of the cleavage was about 10^6-fold lower than efficiency of phosphorylation reaction for all four substrates, as reflected by the K_{cat}/K_m values. The K_{cat}/K_m values of the phosphorylase activities for pyrimidine analogs were very similar, i.e. 3.3×10^{-2} for dCyd and 3.0×10^{-2} for dThd. The degradation of dAdo was three times more efficient then dGuo, i.e.1.1×10^{-2} and 0.4×10^{-2}, respectively. Whereas, *E. coli* PNPase shows more efficient phosphorylase activity for dAdo with a K_{cat}/K_m value of 4.3.[13]

The kinetic parameter of K_{cat} for dThd is half that of dCyd, i.e. 0.044×10^{-2} and 0.024×10^{-2}, respectively. The results reported in Table 2 showed that the most efficient substrate for dCK in the cleavage reaction is dCyd. Our results do not agree with the kinetic parameters of any known nucleoside phosphorylases.[14] The relative specific activity of bacterial pyrimidine nucleoside phosphorylases with dCyd is less then 0.1% compared with dThd.[15]

Discussion

Biochemical processes involving nucleotides and nucleotide precursors are important and fundamental in the life of all cells. One such biochemical reaction is the phosphorolysis of purine and pyrimidine nucleosides, whereby the C–N glycosidic bond is cleaved by a phosphate ion to yield the free nucleobase and P(1)-deoxyribose.[7] There are other reactions responsible for cleaving glycosidic bonds; e.g. nucleoside hydrolases,[16] nucleoside deoxyribosyltransferase,[17] and phosphorybosyltransferase.[18] Deoxycytidine kinase is one of four salvage enzymes and it has broad substrate specificity. It is responsible for the phosphorylation of deoxynucleosides to form the corresponding monophosphates using ATP and UTP as phosphate donors. Here, we present the discovery of the ability of dCK to degrade 2′-deoxyribonucleosides to the respective nucleobases and P(1)-deoxyribose. This unexpected property of dCK was observed during our previous NMR investigations.[4]

One of the aims of this study was to clarify whether the cleavage effect is the result of contamination from bacterial cells or is due to an unexpected dCK activity. There are three enzymes responsible for this type of metabolism in *E. coli*; PNPase, UDPase, and TDPase.

The native enzyme complexes correspond to a homohexamer for UDPase (162 kDa), a homodimer for TDPase (94.6 kDa), and a homohexamer for PNPase (155 kDa).[8–10] Thus, they all have greater molecular sizes as native enzymes then dCK (60 kDa) and the purification procedure used here should lead to minimal contamination of recombinant dCK by the bacterial phosphorylases.

There are other important differences in functional activities as well as substrate specificities between the known nucleoside phosphorylases (NPases) and dCK. All three NPs catalyze the phosphorylytic cleavage of glycosidic bonds reversibly and, in addition to the phosphorolysis reaction, they catalyze a transfer of the deoxyribosyl moiety from one pyrimidine/purine base to another. Our investigations showed that dCK has only the ability to cleave nucleosides with different efficiency. dCK did not catalyze a reversible reaction and did not show any deoxyribosyl transferase activity. Moreover, the products of the phosphorolysis reaction, bases and P(1)-deoxyribose, were both found to inhibit the reaction of the *E. coli* nucleoside phosphorylases. Addition of different nucleoside bases or P(1)-deoxyribose gave no effect on the cleavage efficiency of dCK.

The analysis of the pH optimum for the phosphorolysis activity of dCK (pH 7.5) revealed which

type of inorganic phosphate is likely to be involved, i.e. HPO_4^{2-}, similar to the case of the other NPases.[11,12] It has been reported that the pH optimum for cleavage by TDPase is 6.3, while deoxyribosyl transferase activity has an optimum at pH 7.3. The pH optimum of PNPase appears to reach its optimum activity at pH 8.5 and TDPase activity was characterized by an optimum around pH 6.6. The fact that the cleavage reaction was dependent on inorganic phosphate is a prerequisite for the suggested reaction mechanism. The high pH optimum of dCK indicates further that the observed cleavage reaction is not due to a contamination by *E. coli* NPases.

The sequences of dCK and the known nucleoside phosphorylases present in the protein databases (Entrez and GenBank at NCB) using standard searching methods revealed no significant homology. The crystal structure of dCK was published recently.[19] There are no obvious similarities between the crystal structure of dCK and the known crystal structures of the NPase protein families.

However, it is possible that dCK and NPase share the same catalytic mechanism for cleavage of nucleoside. A diagram of the proposed catalytic mechanisms was presented by Pugmire & Ealick (and see Figure 8).[7,20,21] Probably, the steps beginning with the conformational transition of dCK may lead to weakening of the glycosidic bond due to a high-energy conformation of the bound pyrimidine/purine nucleosides. The phosphate ion, which probably binds on the α-side of the deoxyribose ring may be able to attack the C-1′ position to yield 2-deoxy-α-D-ribose-1-phosphate and the respective nucleobase.

The formation of P(1)-deoxyribose occurred preferentially with reduced cysteine residues of dCK because the reaction required the presence of DTT. The role of the six Cys residues in dCK is not known. Previous investigations showed that reducing agents are necessary for the stability of recombinant dCK but not important for the phosphorylation efficiency.[3,4] The kinetic properties of the enzyme are complex and earlier steady-state and pre-steady-stated kinetic studies have demonstrated the existence of several conformational states of dCK.[6] One possible explanation for phosphorolytic activity is that the enzyme undergoes a conformational change in the presence of phosphate. Probably, reduced cysteine residues are important for the binding of the phosphate ion needed for the phosphorolysis reaction. There is no report that the activity of NPases required or depended on reducing agents such as DTT or mercaptoethanol.

A major concern for the interpretation of the results presented here is the possibility of contaminations of dCK being responsible for the phosphorylytic activity. However, we have here provided a series of arguments for why it is very unlikely. But the main proof for the new type of dCK-catalyzed reaction is the fact that there is no known phosphorylase with the same specificity. dCyd is not cleaved by the nucleoside phosphorylases described so far.[1,15] We have demonstrated here that dCK has the capacity to degrade all natural deoxyribonucleosides but with preferences for pyrimidine nucleosides. Such an ability can be explained by the fact that dCK is an enzyme with broad substrate specificities due to formation of the weakly bound complexes with many different deoxyribonucleosides.[4]

These results are important when dCK is used in phosphorylation experiments *in vitro*, including crystallization and NMR spectroscopy, with high concentrations of the protein or nucleosides, since

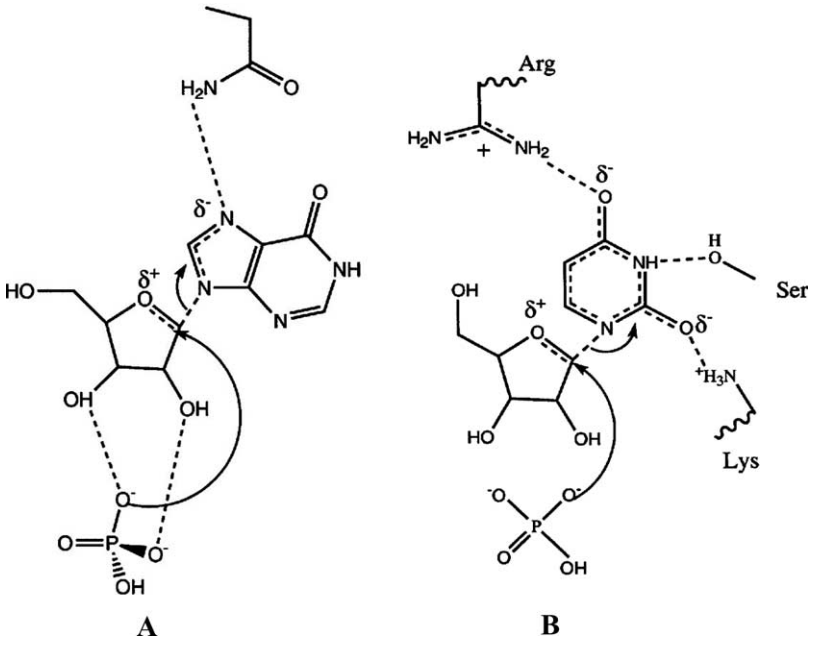

Figure 8. A diagram of the proposed catalytic mechanisms for the human (A) NP-I and (B) NP-II enzyme families as presented by Pugmire & Ealick.[7]

dCK may degrade some nucleosides instead of phosphorylating them.

Materials and Methods

Materials

[5-^{3}H]Deoxycytidine (32 Ci/mmol) and [methyl-^{3}H]-thymidine (51 Ci/mmol) were purchased from Amersham Corp. (Little Chalfont, UK). [2,8-^{3}H]2′-Deoxyadenosine (4 Ci/mmol) and [8-^{3}H]deoxyguanosine (5.8 Ci/mmol) were purchased from Moravek Biochemical Inc (US). Unlabeled nucleosides, ATP, gel-filtration molecular mass standards and chemicals were obtained from Sigma Chemical Co. Restriction grade thrombin protease, vector pET-9d and bacterial cells (JM109, BL21 (DE3) pLysS) were from Novagen (Madison, US). Ni-NTA-agarose was from Qiagen. Ion-exchange paper DE81 was obtained from Whatman International Ltd (UK), and PEI-cellulose FTLC plates were from Merck KGaA (Germany). 2-Deoxy-α-D-ribose-1-phosphate was purchased from Sigma-Aldrich Chemie GmbH (Germany). The structures of labeled nucleosides and reference compounds are shown in Figure 1.

Enzyme preparation and purification

Human dCK was prepared using the pET-9d bacterial vector system. Induction was performed for four hours at 37 °C in the presence of 1 mM IPTG. The bacteria were disrupted by freeze-thawing in 20 mM Tris–HCl (pH 7.9), 0.5 M NaCl, 1 mM PMSF. The lysine was centrifuged at 45,000 rpm for one hour at 4 °C, and dCK was then purified by metal chelate affinity chromatography. Protease cleavage of the His tag was performed with thrombin (Pharmacia Biotech). Cleavage was monitored by SDS-PAGE.[3]

Gel-filtration chromatography

Gel-filtration chromatography was performed using FPLC on a Superdex® 200 column with the Pharmacia Monitor UV-II (Pharmacia Biotech) operating at 280 nm and a flow-rate of 1.5 ml min^{-1} in 10 mM potassium phosphate buffer (pH 7.3). BSA (M_r 66,000) and carbonic anhydrase (M_r 29,000) were used as molecular mass standards. Protein fractions corresponding to 60 kDa were collected and concentrated with the centrifugal filter device Ultrafree®-15 (Millipore). To prepare protein sample for NMR experiments, the 10 mM potassium phosphate buffer in H_2O was exchanged to the same buffer in ^2H_2O where the final concentration of ^2H_2O was 90% (v/v).

Phosphorylation assay

The activity of dCK was followed routinely by a radiochemical assay procedure using tritium labeled substrates as described.[5] The method is based on the measurement of labeled monophosphate product bound to Whatman DE-81 ion-exchange filters. Assays were performed in 50 mM Tris–HCl (pH 7.6) or 10 mM potassium phosphate (pH 7.2) containing 100 mM KCl, 5 mM $MgCl_2$, 5 mM ATP, 2 mM DTT, 0.5 mg/ml BSA. The reaction was initiated by adding recombinant dCK and samples were removed after 10, 20, 30 minutes. K_m and V_{max} values were calculated by fitting velocity data to the Michaelis–Menten equation using non-linear regression analysis provided in SigmaPlot Enzyme Kinetic V.7, 2001. Each value is the mean of at least three separate determinations. A subunit molecular mass of 30,000 was used to calculate the K_{cat}.

Phosphorylase assay

The assay was performed in 10 mM potassium phosphate (pH 7.2) buffer, in some cases in 10 mM Tris–HCl (pH 7.6) buffer, in the presence (or absence) of 10 mM DTT and nucleoside substrates. The reaction was initiated by adding enzyme, followed by incubation at appropriate temperature. After incubation for different lengths of time, 15 µl of the reaction solution was withdrawn and boiled for one minute. The samples were centrifuged and 10 µl of the supernatant was applied to PEI-cellulose FTLC plates.[22] The nucleobases were then separated from the corresponding unreacted nucleosides by chromatography during four hours in 0.1 M H_3BO_3 as the mobile phase. Standards containing the nucleobase and the corresponding nucleosides as markers were used and visualized by UV illumination. The nucleobase and nucleoside spots were excised and eluted with 0.5 ml of a 1 : 1 (v/v) mixture of 0.2 M KCl and 0.1 M HCl, and quantified by liquid scintillation counting.[3] Values of K_m and V_{max} were calculated as described above.

HPLC analysis of nucleosides and nucleobases

HPLC analysis was used to determine the formation of cytosine, adenine, guanine and thymine during the reaction. The nucleobases were separated from the residual 2′-deoxyribonucleosides by reversed-phase chromatography using a C-18 column. The mobile phase was a linear gradient of MeOH in water from 0% to a final concentration of 20% (v/v). The flow-rate was 0.5 ml min^{-1}. The injection volume was 50 µl. All operations, sample injection, control of gradient, UV detection, peak integration and column regeneration were controlled automatically by the HPLC system manager software; the system operated under full automation from sample injection to data report using a CSW32 Chromatographic Station.

NMR experiments

The NMR experiments were carried out on Bruker DRX spectrometers 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, or 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 probe head with triple axis gradients for ^{1}H, ^{13}C, ^{31}P and ^{15}N (QXI). Hard ^{1}H pulses were applied with 29 kHz field strength. ^{13}C hard pulses were applied with 19.2 kHz field strength. ^{13}C decoupling was performed using GARP 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 2.08 kHz applied field. ^{2}H decoupling utilised a WALTZ16 sequence using a 588 Hz field strength.

$^{1}H–^{31}P$ and $^{1}H–^{13}C$-correlation experiments

Standard HSQC type experiments were performed to obtain inverse proton–phosphorus or proton–carbon correlation at the magnetic field strength of 14.1 T, but with modification so that ^{2}H decoupling was applied during the acquisition period. For $^{1}H–^{31}P$ experiments, 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 *F*2 and 30 ppm in *F*1 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 spectra were measured with proton decoupling.

Dipole–dipole (DD) cross-correlation relaxation (CCR) experiments based on the relaxation of multiple quantum coherences of $^{1}H–^{13}C$ were performed as described.[4] Two types of spectra were obtained from a cross and a reference experiment. In a cross experiment, the evolving period $\Delta = 1/2J_{CH}$ of two coupling constants, $J_{Ci,Hi}$ and $J_{Cj,Hj}$, was set up to zero. In a reference experiment $\Delta =$ 3.36 ms was applied, which corresponds to $J_{CH} = 148$ Hz. 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.

For $^{1}H–^{13}C$ correlation experiments, 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 *F*2 and 160 ppm in *F*1 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.

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Supplementary Data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jmb.2004.10.016

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