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Identification of residues involved in the substrate specificity of human and murine dCK

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Abstract

Deoxycytidine kinase (dCK) is a salvage pathway enzyme that can phosphorylate both pyrimidine and purine deoxynucleosides, including important antiviral and cytostatic agents. Earlier studies showed that there are differences in kinetic properties between human and murine dCK, which may explain differences in toxic effects of nucleoside analogs. To determine if certain substitutions in amino acid sequences between human and mouse dCK give these differences in substrate specificity the 14 mutants and hybrid forms of human dCK were studied. All variants were characterised with dCyd, dAdo and dGuo as phosphate acceptors and ATP and UTP as phosphate donor. The relative activities with dCyd, dAdo and dGuo were about 70, 20, 30%, respectively, with UTP as compared to ATP for human dCK and 40, 60, 70% for mouse dCK. Among all tested mutants only the triple combination of substitutions Q179R–T184K–H187N (RKN) had a kinetic behaviour very similar to mouse dCK. The kinetic patterns with several important nucleoside analogs, such as AraC, CdA, ddC and AraG have also been studied. Results demonstrated 50–70% low relative capacities of the recombinant mouse and triple mutant RKN to phosphorylate this nucleoside analogs compare with human dCK. A model for dCK was used to try to explain the functional role of these amino acid substitutions. According to this model the triple mutant RKN have altered amino acids in a region necessary for conformational changes during catalyses. This may affects the substrate selectivity both for the nucleosides and the phosphate donors.

Keywords: Deoxycytidine kinase; Site-directed mutagenesis; Phosphate donor; Substrate specificity

1. Introduction

dCK (EC 2.7.1.74) is a key enzyme in the salvage pathway and responsible for phosphorylation of deoxynucleosides to the corresponding monophosphates using nucleoside triphosphates as phosphate donors. Several pharmacologically important deoxynucleosides are phosphorylated by dCK [1–5], i.e. the cytostatic nucleoside analogs, AraC, 2′2′-difluorodeoxycytidine, CdA [6] and the antiviral nucleoside analogs ddC [7] and AraA [8].

The enzyme has been purified from the several sources and some species comparisons of the properties of dCK have been done [9]. It was found that partially purified mouse dCK had a lower capacity to activate dAdo and ddC as compared with human dCK [10]. This could be one reason for the earlier observed fact that murine lymphoblastoid cell lines accumulate much less dAdo and ddC nucleotides as compared to human lymphoblastoid cell lines, and these analogs are also less toxic to mouse cells than to human cells [11–13].

Human and mouse dCKs have been cloned, expressed and characterised [7,14,15]. Although the amino acid sequences are 88% identical between mouse and human dCK the enzymes have different kinetic behaviour. Both enzymes had a broad substrate specificity phosphorylating many pyrimidine and purine analogs, but the mouse enzyme was less efficient in phosphorylating dAdo, dGuo, ddC as compared to human dCK [15,16].

ATP and UTP, as well as many other nucleoside triphosphates can serve as phosphate donors for dCK. The K_m values for the nucleoside substrates usually decreased when UTP was used as phosphate donor instead of ATP.

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Abbreviations: dCK, deoxycytidine kinase; dGK, deoxyguanosine kinase; CdA, 2-chloro-2′-deoxyadenosine; AraA, arabinosyl adenine; AraC, arabinosyl cytidine; AraG, arabinosyl guanine; ddC, 2′,3′-dideoxycytidine; His-tag, histidine affinity target; IPTG, thio-β-D-galactoside; TAE, Tris–acetate/disodium ethylenediaminetetraacetate.

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Human dCK showed a preference for UTP whereas the mouse enzyme had approximately equal efficiency with both ATP and UTP [16,17]. These results demonstrated that the nature of the phosphate donor apparently affects the substrate specificity of dCK for certain nucleosides but that this effect was variable in dCK from different sources.

In the present investigation we try to determine if certain substitutions in amino acid sequences between human and mouse dCK give these differences in substrate specificity *in vitro*. In order to that series of mutations were introduced in the cDNA for human dCK leading to substitutions mimicking the mouse and/or rat dCK sequence [15]. The mutant proteins were expressed, purified and characterised with regard to the activity with dCyd, dAdo and dGuo using UTP or ATP as substrates. The kinetic patterns with several important nucleoside analogs such as AraC, CdA, ddC and AraG have also been studied. It has been found that only the hybrid with triple combination of substitutions RKN showed very similar kinetic values to those of mouse dCK and clearly different from those with the human enzyme.

A model for dCK was used to try to explain the functional role of these amino acid substitutions. The three-dimensional structure of dCK was based on the recently determined crystal structure of the mitochondrial dGK. These enzymes constitute a family with structural and kinetic properties in common.

2. Materials and methods

2.1. Site-directed mutagenesis

Mutagenesis of the cloned cDNA was effected by PCR where the pET-9d/HdCK construct was used as the template. Site-directed mutagenesis was performed according to Ho *et al.* [18] generating two overlapping mutated fragments and in a second PCR reaction the full length mutated sequence. The cloning primers had the following sequences (where N-terminal—*Nco*I and C-terminal—*Bam*HI restriction sites are indicated with bold letters): forward 5′-CGGAATT**CCATGG**CCACCCCGCCCAAGA-3′; reverse 5′-CAC**GGATCC**TCACAAAGTACTCAAAAACTCTTT-3′. The resulting 780-base pair band was gel-purified and digested with *Nco*I and *Bam*HI (PROMEGA) prior to ligation into the pET-9d vector.

The sequences of the mutagenic oligonucleotides showing in Table 1 were used to for constructing mutants: S8F, P10S, F12P, C45S, M73T, N77S, N164S, Q179R, T184K, H187N, N224S and the triple mutant RKN. Primers are from GIBCO BRL Custom Primers (Life Technologies). The deletion mutant d26, lacking 26 amino acids from C-terminal part of human dCK, was the result of a PCR amplification mistake during preparation of one of the mutants. The mismatched nucleotides are underlined.

The hybrid between human dCK and mouse dCK was constructed with the N-terminal part from human dCK and the C-terminal part from mouse dCK. The restriction site *Mae*III has been used for hybrid preparation. This restriction site corresponds the position 133 in the protein sequences of both human and mouse dCK. The mutations were confirmed by cycle sequencing using the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kits and an ABI prism instrument (ABI Prism® 310 Genetic Analyzer, PE Applied Biosystem, Perklin-Elmer).

2.2. Enzyme preparation and purification

The desired ligation products were transformed first into JM 109 bacterial cells and subsequently into BL21 (DE3) pLysS for protein expression (Novagen). A single plasmid-carrying colony was inoculated in M9ZB growth media containing 25 mg/mL kanamycin and chloramphenicol. Expression of dCK coding DNA was induced by the

Table 1
Oligonucleotide primers used for site-directed mutagenesis

Mutations	Sense primer 5′–3′	Antisense primer 5′–3′
S-8-F	CCCAAGAGATTCTGCCCGTCT	AGACGGGCAGAATCTCTTGGG
P-10-S	AGAAGCTGCCCTTCTTTCTCA	TGAGAAAGAAGGGCAGCTTCT
F-12-P	TGCCCGTCTCCCTCAGCCAGC	CTGGCTGAGGGAGACGGGCA
C-45-S	AAACAATTGTCTGAAGATTGG	CCAATCTTCAGACAATTGTTT
M-73-T	GAACTTACAACGTCTCAGAAA	TTTCTGAGACGTTGTAAGTTC
N-77-S	TCTCAGAAAAGCGGTGGGAAT	ATTCCCACCGCTTTTCTGAGA
N-164-S	TGGATGAATAGCCAATTTGGC	GCCAAATTGGCTATTCATCCA
Q-179-R	ATTTATCTTCGAGCCACTCCA	TGGAGTGGCTCGAAGATAAAT
T-184-K	ACTCCAGAGAAGTGCTTACAT	ATGTAAGCACTTCTCTGGAGT
H-187-N	ACATGCTTAAACAGAATATTA	TAATATTCTGTTTAAGCATGT
N-224-S	CTGAAAACCAGCTTCGATTAT	ATAATCGAAGCTGGTTTTCAG
RKN	ATTTATCTTCGAGCCACTCCAG	ATATATTCTGTTTAAGCACTTC
	AGAAGTGCTAAACAGAATATAT	CTTGGAGTGGCTCGAAGATAAAT

The codons which are responsible for the mutated amino acids are underlined. The triple mutant Q179R–T184K–H187N is denoted with its initial letters for the mutated amino acids: RKN.

addition of IPTG and growth was continued for 4 hr at 37°. Cells were harvested by centrifugation at 2400 *g* (Sorval® RC 3C Plus, DuPont) for 10 min at 4°, resuspended and lysed by freeze-thawing and sonication for three times for 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 260,000 *g* (2330 Ultraspin 55, LKB) for 1 hr at 4° and dCK was then purified by metal chelate affinity chromatography with using Ni^{2+}–NTA agarose resin (Qiagen). 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. Addition of 10 mM DTT and 20% glycerol to the samples were done directly after elution. Protease cleavage was performed with Thrombin as described earlier [19]. The purity of the enzyme and the extent of digestion were determined using SDS–PAGE.

2.3. Enzyme assay using labelled nucleosides

dCK activity was routinely followed by a radiochemical assay procedure as described [19] using the tritium labelled substrates [5-^{3}H]dCyd (Amersham Corp.), [8-^{3}H]dGuo, [2,8-^{3}H]dAdo (Moravek Biochemical Inc.). The method is based on the measurement of tritiated monophosphate product bound to Whatman DE-81 ion exchange filters. Assays were performed in 50 mM Tris–HCl (pH 7.6), 100 mM KCl, 5 mM $MgCl_2$, 5 mM ATP or 5 mM UTP, 2 mM DTT, 0.5 mg/mL BSA, 50 ng of pure dCK and a radiolabelled nucleosides at the concentrations indicated. Analysis of substrate kinetics were done using the Michaelis–Menten equation and non-linear regression analysis with the SigmaPlot 2001, Enzyme kinetic from SPSS Science. Kinetic values were from one experiment, which have been repeated at least twice with very similar results.

2.4. Phosphate transfer assay using [γ^{32}P]-ATP

The adenosine 5′-triphosphate transfer assay was performed with 0.05 μM [γ^{32}P]-ATP (10 μCi/mL) (Amersham Corp.), 100 μM ATP, 50 mM Tris–HCl (pH 7.6), 5 mM $MgCl_2$, 100 mM KCl, 0.5 mg/mL BSA, 10 mM DTT and 100 μM nucleoside analog. The reaction was initiated by adding 100 ng of the enzyme followed by incubation at 37° and terminated by boiled after 25 min. The 2–4 μL of the supernatant was applied to PEI-cellulose FTLC plates (Merck KgaA). Chromatography was performed for 8–12 hr with isobutyric acid:NH_4OH:H_2O (66:1:33, v/v) as the mobile phase. The products of the kinase reaction were detected by autoradiography. The spots were excised and eluted with 0.5 mL of 0.2 M KCl/0.1 M HCl (1:1, v/v) and quantified by liquid scintillation counting [9] or by the phosphoimaging system, Fuji BAS 2500/LAS 1000 (Fujifilm, I&I—Imaging & Information) with an Image Reader V 1.7E. The value obtained with dCyd at 100 μM for each enzyme was set as 1 and phosphorylation of the other substrates were expressed relative to this value.

2.5. 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 the flow rate was 0.4 mL/min. The column was equilibrated and eluted with a buffer containing 20 mM Tris–HCl (pH 7.3), 1 mM DTT and 0.4 M NaCl. BSA (M_r: 66,000) and carbonic anhydrase (M_r: 29,000) were used as molecular weight markers.

3. Results

3.1. Expression and purification of recombinant proteins

After expression and purification the His-tag was effectively removed from the recombinant protein using Thrombin. The purified protein preparations contained a single 30 kDa band in SDS–PAGE analysis. FPLC chromatography showed a single peak of active dCK of approximately 60 kDa MW [19] (data not shown). To avoid the side effects due to different preparations of recombinant proteins, the enzymes, which were used for the investigations, had approximately the same specific activity (not less than 50 nmol/min/mg with 25 μM dCyd). The characterisations were also made only with freshly prepared enzymes.

To increase the stability of dCK during purification reducing agents such as DTT or β-mercaptoethanol are usually added to prevent protein aggregation and inactivation [1]. To determine if DTT can stabilise human and mouse dCK several experiments were done with different combinations of DTT present during the incubation (30 min) and/or in the reaction buffers. We observed that there were considerable differences in the stability of the two dCK enzymes, with mouse dCK being more stable than the human enzyme in this kind of experiments. Preincubation with DTT reactivated human dCK, especially when dAdo was used as substrate. However, the activities of the dCK preparations were not significantly affected by the presence of DTT in the reaction buffers (data not shown).

3.2. Characterisation and kinetic properties of purified enzymes

Fig. 1 shows structural sequence alignment of the human, mouse and rat dCKs. The human dCK sequence is given as the top line and the amino acid substitutions for mouse and rat dCK are given in single letter code. The numbers above show the positions of mutated amino acids.

For screening and identification of the mutants showing kinetic similarities with either human dCK or mouse dCK, we used a standard assay where the relative activities were determined with ATP or UTP (5 mM) as phosphate donors

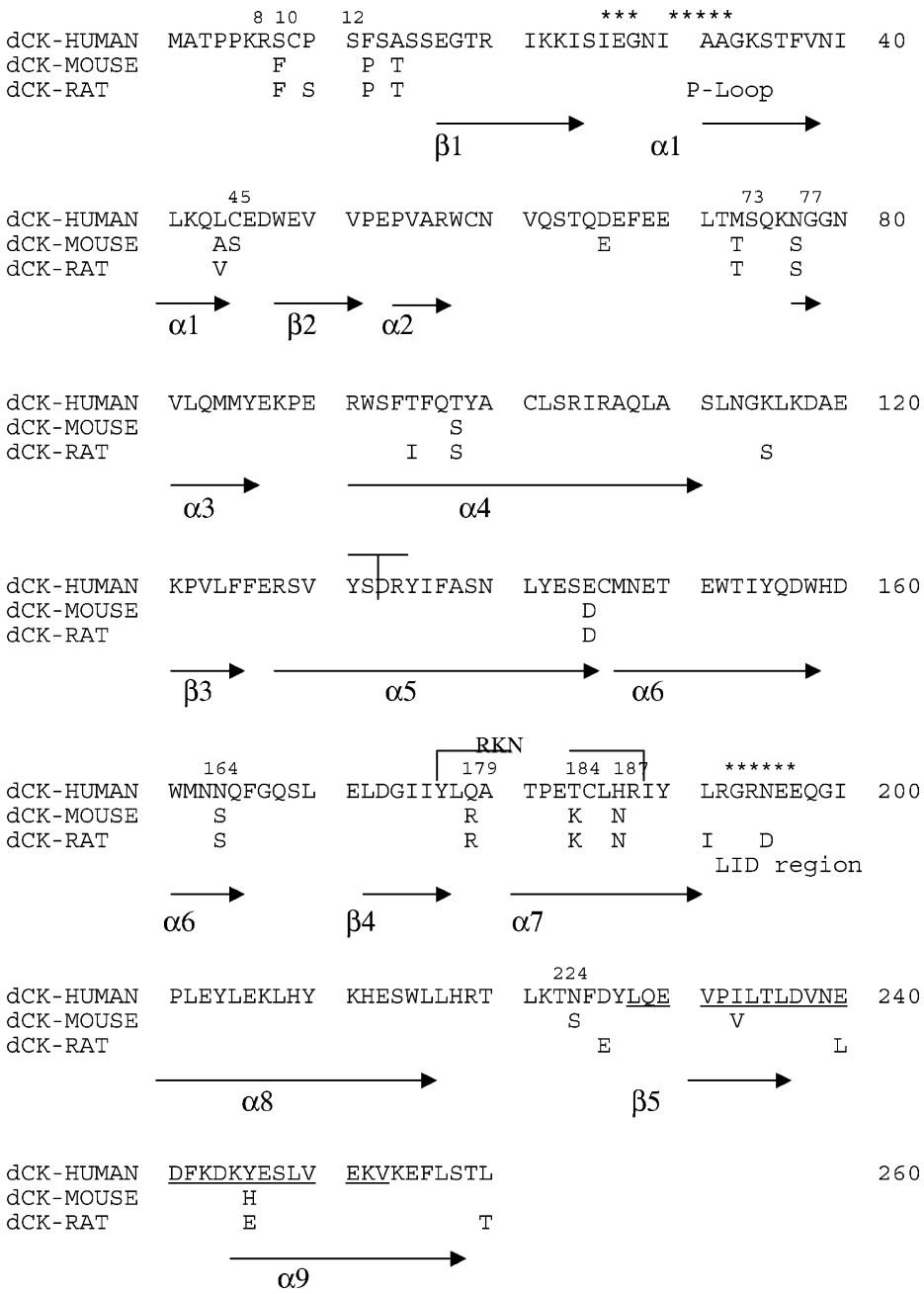

Fig. 1. Structural sequence alignments of human, mouse and rat dCKs. The residues that are identical to the top line (human dCK) are not shown and the letters indicate the amino acid substitutions. The numbers above show the position of mutations produced in this study. The vertical line indicates where the hybrid between human and mouse dCK was made. The amino acids missing in the deletion mutant d26 is underlined. The asterisk (*) indicates the amino acids compose the functionally important sites for deoxyribonucleoside kinases. Arrows indicate alpha-helixes and beta-sheets in the structure model of dCK (see Fig. 4).

and dCyd, dAdo or dGuo (50 μM) as phosphate acceptors. The relative activities with ATP as compare to UTP were then calculated and presented in Fig. 2. The activity with ATP was set as 100%. The results showed that ATP was a better phosphate donor than UTP at 5 mM concentration for the various dCK preparations.

Human recombinant dCK had higher activity for purine nucleosides with ATP as phosphate donor as compared to when UTP was used. In case of recombinant mouse dCK the activities for these nucleosides with ATP or UTP were not significantly different. In case of dAdo and dGuo phosphorylation the relative activities with UTP as compared to ATP were 20 and 30%, respectively, for the human enzyme and 60 and 70%, respectively, for the mouse enzyme. But the activity with dCyd as acceptor was about 70% with UTP for human dCK and 40%, respectively, for mouse dCK. These differences in kinetic properties were used for characterisation of the mutants and since there was approximately ±10% SD in the measurements only relative differences larger than 15% were considered significant.

The hybrid HdCK/MdCK had the N-terminal part from human dCK and the C-terminal part from mouse dCK

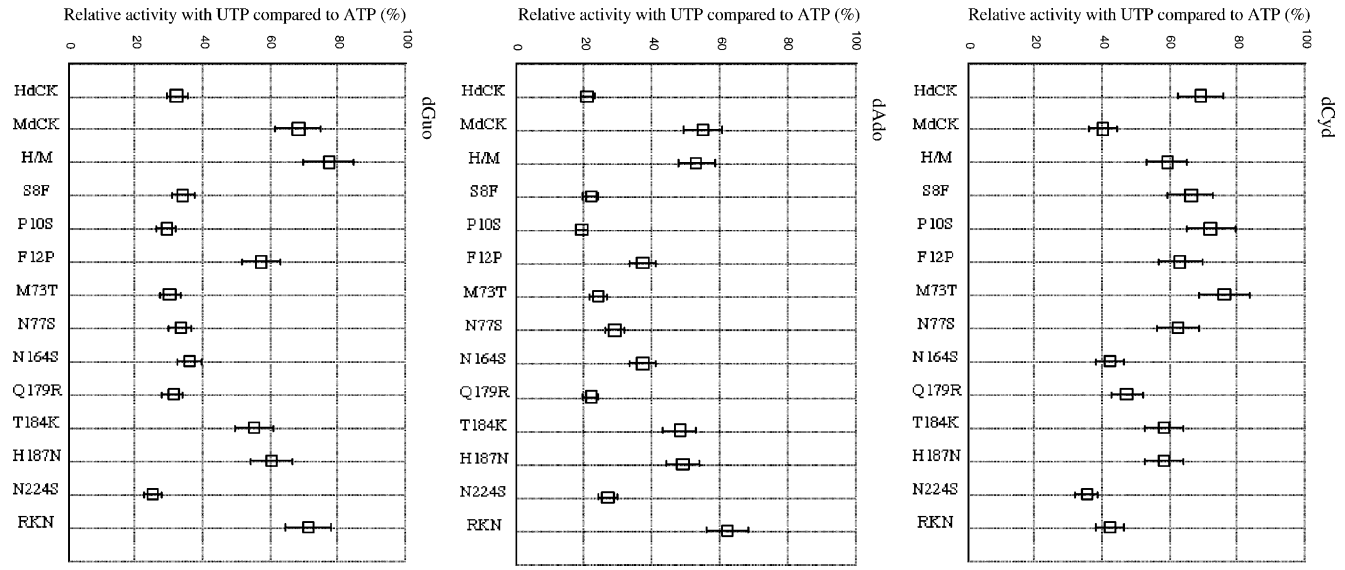

Fig. 2. Relative activity with UTP compared to ATP for different substrates. The assay for human, mouse and various mutant forms of dCK was performed by the radiochemical method as described in Section 2. Phosphorylation efficiency for each enzyme with UTP was relative to that with ATP which was set to 100% respective to each substrate. The concentration of nucleosides was 50 μM and 5 mM for ATP and UTP as well. The values (nmol/min/mg) are the means of at least three different determinations and the SD was ±10%. HdCK: human dCK; MdCK: mouse dCK; H/M: the hybrid between human dCK and mouse dCK; RKN: the triple mutant RKN.

(Fig. 1) and this enzyme showed a increased relative activity with dAdo and dGuo but not with dCyd, using UTP as phosphate donor, as compared to the human wild type enzyme (Fig. 2).

We observed that the point mutations S8F, P10S, M73T, N77S behaved similar to human wild type dCK with all three substrates. There are small changes the activity for F12P mutant with dAdo and somewhat with dGuo. Enzymes with point mutations N164S or N224S had very similar properties and they seemed to influence the activity with dCyd but somewhat less the activity with dAdo and dGuo (Fig. 2). Enzyme with mutation Q179R showed reduced activity with dCyd but not with the two other substrates. Two other point mutations in the same region, T184K and H187N, had significant changes in the activities with dAdo and dGuo. However, only the triple mutant RKN showed an activity profile very similar to that of the mouse enzyme (Fig. 2).

As described above the single mutation in position S8F in the N-terminal did not lead to major changes in the activity profile but it showed an increased stability during purification and storage. The lack of one of six cysteines in the mutant C45S led to an unstable enzyme that could not be purified and characterised. The d26 C-terminal deletion mutant had lost about 95% of its activity with all three substrates but it still eluted in the position of a dimer in gel filtration chromatography (data not shown).

3.3. Kinetic parameters of human dCK, mouse dCK and triple mutant RKN

As was noted above, the three mutants F12P, N164S and N224S have the same changes in relative activities with one substrate: dGuo (F12P) and dCyd (N164S, N224S). However, our results showed that there were no significant differences between kinetic parameters, e.g. the K_m and V_{max} values of the mutants and the human wild type dCK (data not shown). The results in Fig. 2 demonstrated that only the triple mutant RKN was similar to a mouse dCK with all three substrates and its kinetic behaviour was investigated further and compared to both human and mouse wild type enzymes (Table 2). The K_m values for dCyd, dAdo and dGuo with human dCK using UTP were much lower as compare when ATP was the donor. The efficiency ($K_{ef} = V_{max}/K_m$) was clearly higher with UTP for dCyd and dAdo but not for dGuo where the efficiencies for ATP and UTP were similar. Mouse dCK had similar K_m values for dCyd with both donors and there was little difference in the efficiencies. However, the efficiency for dGuo was more then twice higher with UTP. The kinetic values for the mutant RKN were very similar to those of mouse dCK and clearly different from those with the human enzyme (Table 2).

3.4. The activity of human, mouse and some mutant dCKs with different nucleoside analogs

In order to test if the observed activity changes have effects on the substrate specificity with some pharmacologically important nucleosides, we used the phosphate transfer assay with various concentrations of dCyd, ddC, AraC, CdA, AraG and Thd as substrates and [γ^{32}P]-ATP as phosphate donor. Fig. 4 shows the relative activity of human dCK, mouse dCK and the mutant RKN. The activity with dCyd at 100 mM was set to 1.0 for each enzyme separately and the activities with the other sub-

Table 2
Kinetic parameters of recombinant human dCK, mouse dCK and triple mutant RKN

Substrate	Phosphate donor	Human dCK			Mouse dCK			RKN		
		K_m [a]	V_{max} [b]	K_{ef} [c]	K_m	V_{max}	K_{ef}	K_m	V_{max}	K_{ef}
dCyd	ATP	1.5	68	45	3.8	223	59	4	178	45
	UTP	0.3	27	90	3.5	175	50	3.8	118	31
dAdo	ATP	160	1280	8	307	1167	4	267	748	3
	UTP	41	410	10	138	635	5	118	531	5
dGuo	ATP	120	360	3	87	282	3	76	260	3
	UTP	44	160	4	24	191	8	27	184	7

Phosphorylation was followed by the radiochemical assay with tritium labelled nucleosides and 5 mM ATP or UTP as phosphate donor. Kinetic values are from one experiment which have been repeated three times with very similar results and SD is approximately ±10%.
[a] K_m (M).
[b] V_{max} (nmol/min/mg).
[c] $K_{ef} = V_{max}/K_m$.

strates at different concentrations were expressed relative to this value. This was done since the absolute specific activity differed substantially between the enzyme preparations (see Fig. 4).

The concentrations of a nucleosides used in these experiments were from 6 to 100 μM and in case of AraC and CdA half-maximal saturation of the reaction was within this interval. However, with ddC and AraG substrate saturation

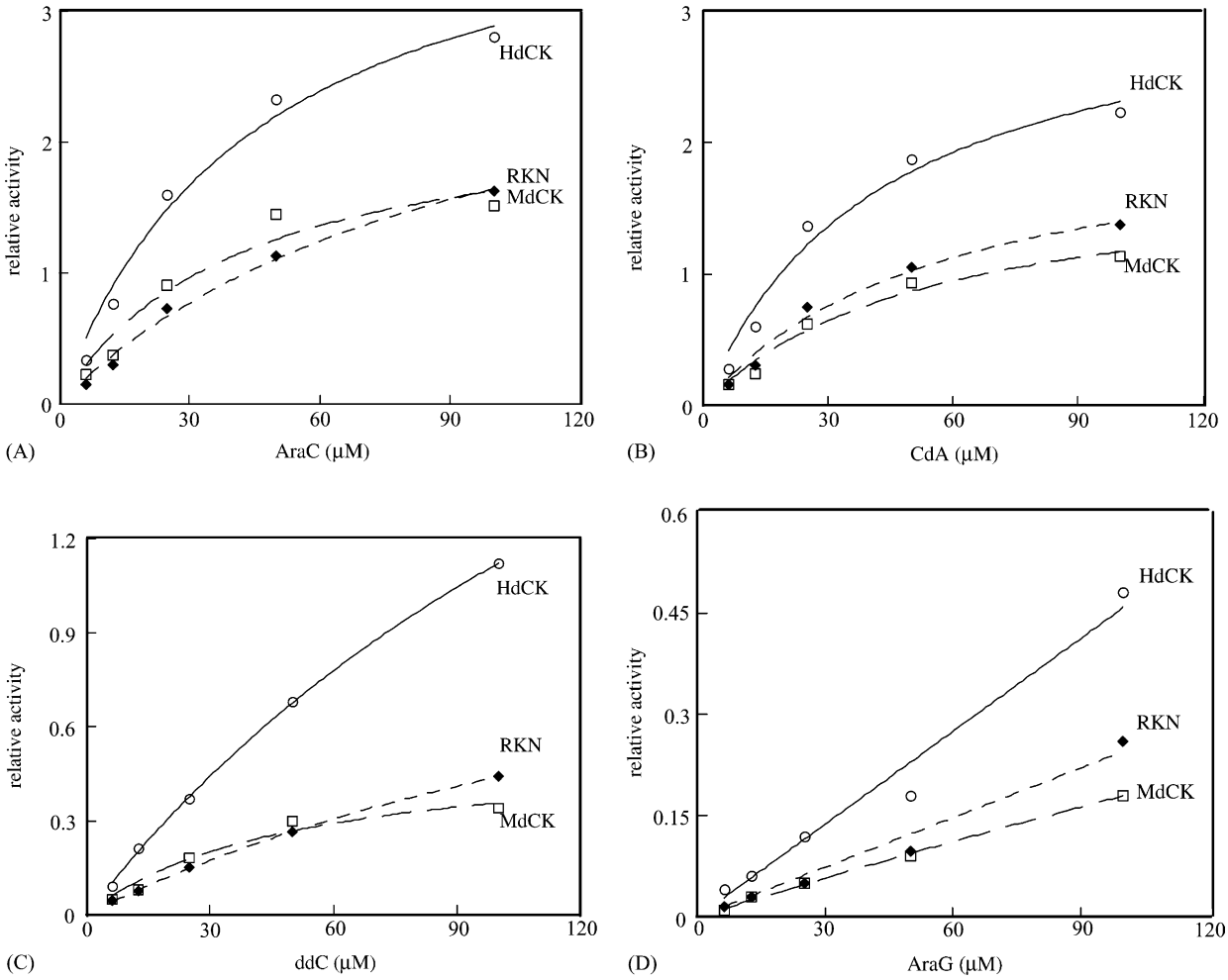

Fig. 3. Kinetic pattern for phosphorylation of nucleoside analogs. Concentration curves for phosphorylation of AraC (A), CdA (B), ddC (C) and AraG (D) with 100 μM [γ^{32}P]-ATP as phosphate donor with human dCK, mouse dCK and the triple mutant RKN. The relative activities of each enzyme at different concentration of nucleosides compared to with dCyd (100 μM) were calculated as described in Section 2. The specific activities with 100 μM dCyd were for human dCK: 53 nmol/min/mg; mouse dCK: 78 nmol/min/mg and mutant RKN: 129 nmol/min/mg. The values are means ± 10% of three independent experiments.

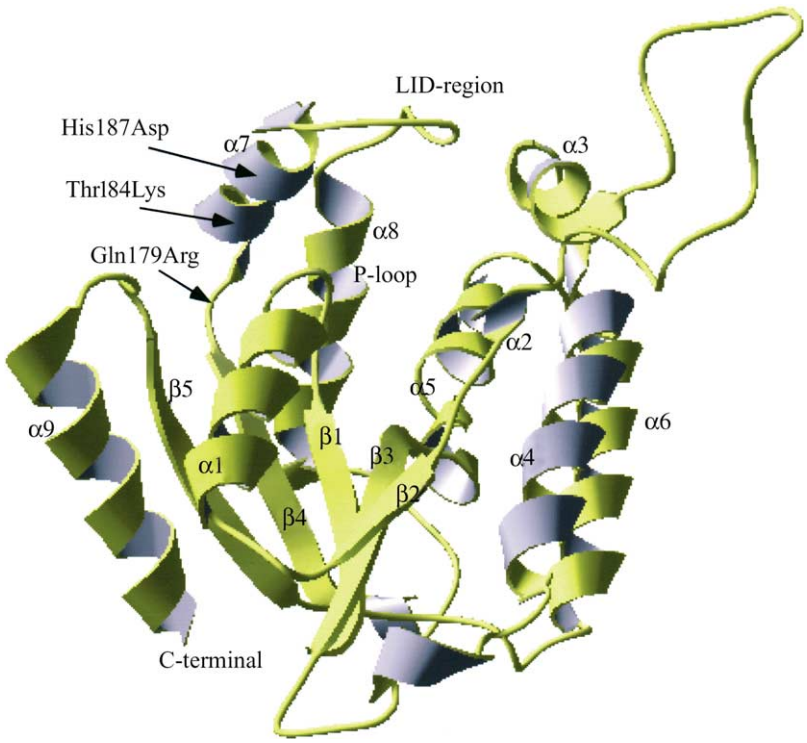

Fig. 4. The structure-model of human dCK. The model is derived on the base of a structure sequence alignment of the dGK–dCK sequence as described by Johansson *et al.* [20]. The numbering of the alpha-helixes and beta-shits correspond to the ones in the Fig. 1. Arrows show the positions of amino acid substitutions in the triple mutant RKN.

was not achieved. Therefore, we cannot calculate apparent K_m and V_{max} values from this data but in the following we are comparing the activities at the two highest concentrations in each case. The order of activity with the different analogs and all four enzymes were as follows: AraC = CdA > ddC > AraG > Thd (data for Thd not shown) (Fig. 4). Human dCK was able to phosphorylate all substrates very efficiently with the exception of AraG, which was used less than 50% as well as dCyd (Fig. 3D). The nucleoside analogs CdA, ddC and AraC showed several fold higher activity with human dCK than with the mutant RKN or mouse dCK (Fig. 3A–C). The activity with ddC was similar to that with dCyd in case of human dCK and 70% lower with mouse and mutant RKN (Fig. 3C). The relative activity with AraG was 15–20% of that with dCyd with mouse and mutant RKN (Fig. 3D). Thus, mouse dCK and mutant RKN showed very similar and several fold lower activities with all the analogs at the concentrations tested.

4. Discussion

In this study, 14 mutants and hybrid forms of human dCK were studied in order to identify amino acid residues involved in substrate recognition. The mutations were chosen based on sequence analyse of dCK from different species (human, mouse and rat are shown on Fig. 1), and a structural sequence alignment of the dNK–dCK–dGK–TK2 family based on the crystal structure of human dGK recently published by Johansson *et al.* [20]. The over all structures of human dGK and dNK were surprisingly similar to that of HSV-1-TK. A model for dCK was used for identification of the presumed active sites of dCK. From the data shown by Johansson we observed that the amino acids involved in nucleotide substrate interactions and in the surroundings of phosphate donor in case of dNK, dGK, HSV-1-TK and probably also for dCK (Fig. 1) are highly conserved. Therefore, we chose non-conservative substitutions in mouse and/or rat dCK, compared with the human enzyme, which can be involved, directly or indirectly, in the binding of the substrates. The amino acids substitutions were produced starting with the human sequence and analysing for their effect on enzyme activity.

The results presented here indicated that it is not the absolute activities of the recombinant mouse dCK vs. the human dCK with the different nucleoside that is important, but rather the relative activities with different substrates. The situation is complicated by the fact that the efficiency of a certain nucleoside is affected by the nature of the phosphate donor. Therefore, we compared the activities of recombinant mouse dCK and human dCK and the mutants with UTP and ATP as phosphate donors and dCyd, dAdo and dGuo as phosphate acceptors.

It was observed that the point mutations S8F, P10S, M73T, N77S did not led to significant alterations in the kinetic properties of human dCK. However, the S8F mutant showed an increasing stability. The point mutations F12P, T184K and H187N showed an altered activity profile for

purine nucleosides similar to that of the mouse enzyme. There were also changes in the dCyd activity of the N164S and N224S mutants. We investigated the kinetic parameters for the triple mutant RKN in comparison to the two wild type enzymes and in case of human and mouse dCK our results are similar to earlier studies [15,16]. Among all tested mutants only the triple combination of substitutions had a kinetic behaviour very similar to mouse dCK.

Also the differences in substrate saturation curves for human, mouse and mutant RKN was tested with the pharmacologically interesting nucleoside analogs ddC, AraC, CdA and AraG at various concentrations. In this case the phosphoryl transfer activity was measured and thus only [γ^{32}P]-ATP could be used as phosphate donor at relatively low concentrations. The overall results clearly demonstrate a reduced relative capacity of the recombinant mouse and the mutant RKN to phosphorylate these nucleoside analogs compared to human wild type. These results may help to explain earlier investigations showing a lower relative sensitivity of mouse cells or whole animals as compared to human cells to ddC, CdA and AraG [11–13].

Fig. 4 shows the dCK model based on the dGK structure and there appears to be no difference in the side chains lining the substrate pocket with only one exception, Ala100 in dCK is Ser114 in dGK about 4 Å from the nucleoside binding site [20]. Figs. 1 and 4 show the positions of the amino acid substitutions in the triple mutant. The alteration Q179R is located at the end of the β4-sheet and the two other substitutions, T184K and H187N, are located in the α7-helix. It has been proposed that this site can be involved in binding of the phosphate donor and could thus influence the kinetic behaviour of enzymes. The γ and β phosphates of ATP or UTP probably interact with the highly conserved Ala32, Lys34, Ser35, Thr36, located in the P-loop, and the two arginins—Arg192 and Arg194, in the LID-region. This region of dCK probably moves during the reaction and covers the active site when the phosphate donor interacts with the 5′-deoxyribose of the nucleoside substrate. According to this model only the triple mutation RKN has altered amino acids in a region necessary for conformational changes during catalyses. This may affect the substrate selectivity both for the nucleosides and the phosphate donor.

From the model structure of dCK it appears that the mutation F12P in the N-terminal part near beginning of β1-sheet of dCK cannot be involved in binding of nucleosides or phosphate donors but may indirectly influence the substrate specificity.

Thus, the region between Gln179 and Gln199 is most likely involved in binding of the phosphate donors by forming the top of the active site. The interactions occurring in this region can probably determine the efficiency of the nucleoside as acceptor via an induced fit mechanism. Substitutions in the dCK sequence in this region seem to give a subtle but definite shift in preference for a certain nucleoside as substrate.

Due to the rate limiting role of dCK for the activation of many analogs, this can lead to an altered capacity of human cells in comparison with mouse cells to accumulate the nucleotide metabolites and thus to a different cytotoxic effect of the analog. One implication of these results is that it could be important to compare the sequences of dCK in this region from the different animals used in drug development. Such studies are now in progress and hopefully the results will help to establish better animal models for future drug testing.

Acknowledgments

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