Pergamon

Biochemical Pharmacology, Vol. 50, No. 2, pp. 163–168, 1995.

0006-2952/95 $9.50 + 0.00

0006-2952(95)00129-8

DIFFERENCES IN KINETIC PROPERTIES OF PURE RECOMBINANT HUMAN AND MOUSE DEOXYCYTIDINE KINASE

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(*Received* 28 *November* 1994; *accepted* 3 *March* 1995)

Abstract—Human and mouse deoxycytidine kinase (dCK) (EC 2.7.1.74) were cloned and expressed in *Escherichia coli*. Michaelis–Menten kinetics were determined for the purified enzymes with 2′-deoxycytidine (dCyd), 2′-deoxyadenosine (dAdo), 2-chloro-2′-deoxyadenosine (CdA), 2′,3′-dideoxycytidine (ddCyd) and 9-β-D-arabinofuranosylguanine (araG) as substrates and ATP and UTP as phosphate donors. Both human and mouse dCK showed highest affinity to dCyd with K_m values of 0.05–0.2 μM. The anti-leukaemic compound CdA was the superior substrate of the nucleoside analogues tested. Both enzymes were able to efficiently utilize ATP and UTP as phosphate donors. However, the use of UTP instead of ATP as phosphate donor decreased K_m values for all substrates investigated. The kinetic properties of mouse and human dCK differed in that the human enzyme showed higher affinity for the substrates dAdo, CdA, ddCyd and araG. The human enzyme also showed higher affinity for ATP and UTP. The ability to phosphorylate dCyd was, however, similar for both human and mouse dCK. At physiological concentration of the feedback inhibitor dCTP, mouse dCK showed lower activity than human dCK for all substrates investigated.

Key words: deoxycytidine kinase; nucleoside phosphorylation; nucleoside analogues; nucleoside metabolism; pyrimidine metabolism; purine metabolism

dCK† (EC 2.7.1.74) is an enzyme with broad substrate specificity phosphorylating the pyrimidine dCyd, the purines dAdo and dGuo, as well as many nucleoside analogues [1]. There exist in the literature both *in vivo* and *in vitro* studies on differences in substrate phosphorylation by human and mouse dCK [2, 3]. Several factors determining enzyme activity must be considered when comparing data obtained by *in vivo* studies with results from *in vitro* assays. In addition to the affinity of the substrate, the presence of several potential phosphate donors and the enzyme inhibitor dCTP will affect the overall phosphorylation rate of a specific substrate. The importance of UTP as the major phosphate donor was first reported by White and Capizzi [4] and was further investigated by Shewach *et al.* [5]. These reports concluded that UTP was the most important phosphate donor for dCK among the different triphosphates tested.

We have recently cloned mouse dCK and expressed both mouse and human dCK in the same expression system to enable the enzymes to be compared [6]. The amino acid substitutions between human and mouse dCK are outlined in Table 1. Although the enzymes amino acid sequences are very homologous between the species, they behave differently in their affinity for the substrates. We have previously reported differences between mouse and human dCK using single substrate concentrations in a phosphoryl transferase assay [6]. In the present study we have determined Michaelis–Menten kinetics for dCyd, dAdo, CdA, ddCyd and araG for both human and mouse dCK. We have further investigated the role of ATP and UTP as phosphate donors for both enzymes, as well as the effect of the feedback inhibitor dCTP. This is the first thorough study of the kinetics of pure recombinant mouse and human dCK. In conclusion we found that (i) all substrates investigated, except for CdA with ATP as phosphate donor, had higher affinity for human dCK than mouse dCK; (ii) human dCK had higher affinity than mouse dCK for the phosphate donors ATP and UTP; (iii) CdA was the most efficient nucleoside analogue tested as substrate for both human and mouse dCK; and (iv) the inhibition of enzyme activity by physiological concentrations of dCTP was more pronounced for mouse dCK than human dCK.

Our results help elucidate previous reports on differences in dCK activity in human and mouse cells and should be considered when evaluating the metabolism of dCK phosphorylated substrates in these different species.

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† Abbrevations: dCK, deoxycytidine kinase; dCyd, 2′-deoxycytidine; dAdo, 2′-deoxyadenosine; dGuo, 2′-deoxyguanine; CdA, 2-chloro-2′-deoxyadenosine; ddCyd, 2′,3′-dideoxycytidine; araG, 9-β-D-arabinofuranosylguanine; PMSF, phenylmethylsulphonyl fluoride.

MATERIALS AND METHODS

Materials. 2′-[5-^{3}H]Deoxycytidine (32 Ci/mmol) was purchased from Amersham Corp. (U.K.) and 2′-[2,8-^{3}H]deoxyadenosine (34 Ci/mmol), 2′,3′-[5,6-^{3}H]dideoxycytidine (5 Ci/mmol), 2-chloro-2′-

Table 1. Physical properties of the amino acid substitutions between human and mouse dCK predicted amino acid sequence

Amino acid residue number	Human dCK	Mouse dCK	Comparison of physical properties of amino acid substitutions*
8	S	F	P to H
14	A	T	N to P
44	L	A	H to N
45	C	S	N to P
66	D	E	− to −
73	M	T	H to P
77	N	S	P to P
98	T	S	P to P
145	E	D	− to −
164	N	S	P to P
179	Q	R	P to +
184	T	K	P to +
187	H	N	P to P
224	N	S	P to P
233	I	V	H to H
246	Y	H	P to P

* Abbreviations: N, neutral; H, hydrophobic; P, polar; −, negatively charged; +, positively charged.

[8-^{3}H]deoxyadenosine (20 Ci/mmol) and 9-β-D-[^{3}H]-arabinofuranosylguanine (6 Ci/mmol) from Moravek Biochemicals Inc. (U.S.A.). Unlabelled CdA was kindly provided by Z. Kazimierczuk (Dept. of Biophysics, Univ. of Warsaw, Warsaw, Poland) and unlabelled AraG was kindly provided by Dr G. Koszalka (Burroughs Wellcome). Other unlabelled nucleosides and chemicals were obtained from Sigma. All reagents were of highest purity available.

Enzyme preparation and purification. Human and mouse dCK were expressed using the QIAexpress System (Qiagen). The cDNA sequence was ligated into a pQE vector coding for a histidine affinity tag upstream to the insert. The pQE expression construct was transformed into M15[pREP4] host strain and transformants were selected. A positive colony was inoculated in a large-scale expression culture (LB-broth containing 100 μg/mL ampicillin and 25 μg/mL kanamycin). When OD_{600} reached 0.7, protein expression was induced by addition of 1 mM isopropyl-1-thio-β-D-galactopyranoside and growth continued for 4 hr. The bacterial pellet was lysed by freeze-thawing and sonication 3 × 1 min on ice in 50 mM sodium phosphate pH 8.0, 300 mM NaCl, 5 mM β-mercaptoethanol and 1 mM PMSF. The supernatant was cleared by centrifugation at 10,000 *g* for 20 min at 4° and loaded on the Ni-nitrilotriacetic acid resin provided by the QIAexpress system. Protein was eluted by a stepwise increasing concentration of imidazole (0–0.3 M) in 50 mM sodium phosphate pH 6.0, 300 mM NaCl, 10% glycerol, 5 mM β-mercaptoethanol and 1 mM PMSF. Immediately after the protein was eluted from the column 10 mM dithiothreitol was added to the sample. The purity of the enzyme was verified by SDS-electrophoresis (Phast system, Pharmacia, Uppsala, Sweden).

Enzyme assay. dCK activity was measured using a radiochemical method described by Ives *et al.* [7]. The assay is based on measurement of tritiated monophosphate product bound to Whatman DE-81 ion exchange filters. The assays were performed in 50 mM Tris pH 7.6, 100 mM KCl, 5 mM $MgCl_2$, 15 mM NaF, 5 mM dithiothreitol, 0.5 mg/mL BSA and indicated concentrations of ATP or UTP. Nucleosides and enzyme were added to a total reaction volume of 50 μL. At 15, 30 and 45 min incubation at 37°, 10 μL of the reaction mixtures were spotted on Whatman DE-81 filters. The filters were washed three times in 5 mM ammonium formate. The filter-bound monophosphates were then eluted in 0.2 M KCl and 0.1 M HCl and determined by scintillation count. The amount of enzyme added to the reaction was adjusted so that no more than 15% of the substrate was consumed during the incubation period. All assays were performed in duplicate. The kinetic data were analysed using Enzfitter V1.05 (Leatherbarrow RJ, Elsevier Biosoft 1986).

RESULTS

Kinetic properties of human and mouse deoxycytidine kinase

The Michaelis–Menten kinetic constants of recombinant human and mouse dCK for dCyd, dAdo, CdA, ddCyd and araG are presented in Table 2. All determinations were performed using 5 mM ATP or UTP as phosphate donor. Although non-hyperbolic kinetics for dCyd and dAdo have been reported [8, 9], no deviation from Michaelis–Menten kinetics was observed in the substrate concentration range assayed in this study. Both human and mouse dCK showed highest affinity to dCyd with K_m and V_{max} values similar for both enzymes. The other natural substrate investigated, dAdo, showed 3000-fold higher K_m than dCyd using ATP as phosphate donor. When UTP instead of ATP was used as phosphate donor for dAdo phosphorylation, the K_m

Table 2. The kinetic properties of human and mouse dCK

	Substrate	Phosphate donor (5 mM)	K_m (μM)	V_{max} (nmol/mg/min)	V_{max}/K_m	$V_r = V_{max}/V_{dCyd}$*	V_r/K_m (1/mM)
Human dCK	dCyd	ATP	0.16	33	210	1	6300
		UTP	0.050	16	320	0.48	9600
	dAdo	ATP	480	1500	3.2	47	98
		UTP	19	90	4.7	2.7	140
	CdA	ATP	24	760	31	22	900
		UTP	0.99	120	120	3.5	3500
	ddCyd	ATP	230	22	0.10	0.69	3.0
		UTP	150	29	0.19	0.91	6.0
	AraG	ATP	3800	130	0.033	3.7	0.96
		UTP	2000	85	0.043	2.4	1.2
Mouse dCK	dCyd	ATP	0.18	41	230	1	5600
		UTP	0.10	21	210	0.51	5100
	dAdo	ATP	550	970	1.8	24	44
		UTP	150	480	3.2	12	78
	CdA	ATP	17	2220	130	13	790
		UTP	6.3	2400	380	14	2300
	ddCyd	ATP	670	220	0.33	2.0	3.1
		UTP	480	610	1.2	5.7	12
	AraG	ATP	5900	830	0.14	5.0	0.85
		UTP	3300	1700	0.53	11	3.2

* dCyd phosphorylation was used as internal standard to determine the amount of active enzyme in each assay.

decreased 25-fold for human dCK and 4-fold for the mouse enzyme.

Among the nucleoside analogues tested, CdA proved to be the superior substrate for both human and mouse dCK. CdA showed approximately 20–30-fold higher affinity to the enzymes than dAdo. Its kinetic pattern was similar to that of dAdo with a 25-fold decrease in human dCK K_m with UTP rather than ATP as phosphate donor. When UTP was used as phosphate donor the K_m for human dCK was 6-fold lower than for mouse dCK. The affinity for ddCyd was 3-fold higher for human dCK than mouse dCK. The decrease in K_m when UTP was used instead of ATP as phosphate donor was less marked for ddCyd than for the adenosine nucleosides dAdo and CdA. AraG was a very low affinity substrate with K_m values of 2–6 mM, 20,000–40,000-fold higher than for dCyd.

Since an accurate determination of the V_{max} value is dependent on the amount of active enzyme present in a protein preparation we used the phosphorylation of dCyd as an internal standard in the assay, to ensure that the purified proteins were not inactivated or degraded by storage. V_{max} values related to the dCyd internal standard are presented as V_r in Table 2. The V_r/K_m is the most accurate expression of enzyme efficiency. A comparison of the V_r/K_m values of the nucleosides tested shows that even though dCyd phosphorylation showed low V_{max} values compared with other substrates, dCyd exhibited the highest efficiency expressed as V_r/K_m. CdA was the most efficient substrate after dCyd. All substrates showed higher V_r/K_m with UTP as phosphate donor, except mouse dCK with dCyd as substrate.

Previous kinetic determinations of tissue purified human dCK with ATP as phosphate donor showed dCyd K_m of 0.3–2.8 μM [2, 10–14]; dAdo K_m of 21–780 μM [2, 10–14] and ddCyd K_m of 30–280 μM [10, 11]. dCyd K_m was in this study determined to 0.16 μM for recombinant human dCK with ATP. dAdo and ddCyd K_m, 480 μM and 230 μM, are within the range of what previously have been reported for human dCK.

The affinity of UTP versus ATP as phosphate donor for human and mouse dCK

We have further determined the affinity of the phosphate donors ATP and UTP to human and mouse dCK (Table 3). The human enzyme showed higher affinity than mouse dCK to both ATP and UTP. Both enzymes had a preference for UTP, but mouse dCK had only a 1.4-fold higher affinity to UTP whereas the human enzyme showed 8-fold higher affinity for UTP than ATP.

The inhibitory effect of dCTP

Inhibition by dCTP was investigated at 13 μM dCTP (Fig. 1). This is the physiological dCTP concentration in MOLT-4 T-lymphoblasts [5]. Substrate concentrations were set to 5 μM: 50 times the K_m value for dCyd, close to K_m values for CdA, but much lower than K_m for the other substrates. Therefore, the inhibitory effects of different substrates should not be compared, but only the differences in inhibition of human and mouse dCK for the same substrate. When the two enzymes were compared, mouse dCK was more efficiently inhibited by dCTP for all substrates. With ATP as phosphate donor the inhibition of dCyd phosphorylation by 13 μM dCTP was only 30% for human dCK and 50% for the mouse enzyme. The other substrates were more effectively inhibited by dCTP. The enzyme activity was less inhibited by dCTP when UTP instead of ATP was used as phosphate donor in the assay.

DISCUSSION

We have investigated several factors which may determine differences in activity of human and mouse dCK. Both enzymes can efficiently use both ATP and UTP as phosphate donors. A comparison of the affinity of the phosphate donors showed that human dCK had preference for UTP, whereas the mouse enzyme had approximately equal affinity to both ATP and UTP. In intact cells phosphate donor concentrations were determined to be 4.0 mM ATP and 1.5 mM UTP [5]. As the physiological concentrations of ATP and UTP are 100-fold higher than determined K_m, the enzymes are likely to be saturated with phosphate donors *in vivo*. It is therefore possible that UTP is the most important phosphate donor to human dCK, whereas UTP together with ATP are the physiological phosphate donors to mouse dCK. Differences in ATP/UTP ratios between human and mouse cells may, however, also contribute to the physiological effects.

dCTP is a potent inhibitor of human dCK with ATP as phosphate donor [8, 13, 15]. However, more recent studies suggest that the inhibitory effects are reversed by the presence of UTP [4, 5]. As dCTP inhibition is dependent on the phosphate donor, it is possible that the degree of inhibition is determined by the enzyme's utilisation of either ATP or UTP. As human dCK exhibited higher UTP affinity, it may therefore be less inhibited by dCTP than the mouse enzyme. Consequently, the combination of differences in dCTP inhibition together with differences in phosphate donor affinity may add to the differences in nucleoside kinetic properties.

The nucleoside K_m of the enzymes decreased significantly when UTP was used as phosphate donor rather than ATP. The greatest affinity increase was for human dCK with dAdo and CdA as substrates. Mouse dCK had lower affinity to both substrates independent of the phosphate donor. Our data show that human dCK is more capable of converting dAdo and related analogs to their monophosphate products, confirming the results of previous studies on dAdo metabolism [16].

Several studies have concluded that dCK is responsible for the initiation of ddCyd metabolism [17, 18]. When comparing human and mouse cells, significantly higher rates of ddCyd phosphorylation have been observed in human cells [3]. In this study we have shown that human dCK has 3-fold higher affinity for ddCyd than mouse dCK. This could explain the differences observed *in vivo*, although, differences in dCTP inhibition may also contribute.

It is clear that the complex interaction of multiple factors contributes to the differences in dCK-

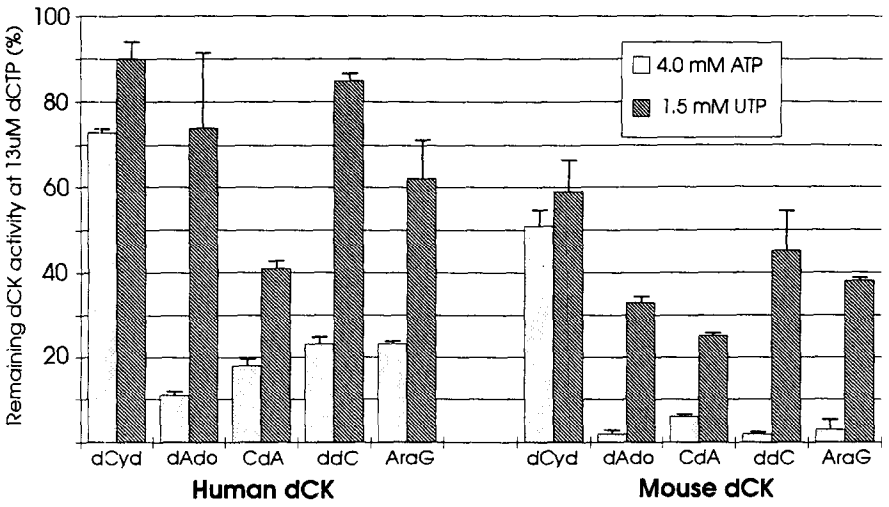

Fig. 1. Remaining dCK activity at 13 μM dCTP and 5 μM substrate concentrations. The amounts of human and mouse dCK added to the assay showed equal levels of dCyd phosphorylation, indicating equal amounts of active enzyme.

Table 3. The kinetic properties of the phosphate donors ATP and UTP (5 μM dCyd and 5 mM of the nucleotides were used in the assays)

Enzyme	Phosphate donor	K_m (μM)
Human dCK	ATP	22
	UTP	2.8
Mouse dCK	ATP	31
	UTP	22

mediated nucleoside metabolism in human and mouse cells. A combination of differences in phosphate acceptor and phosphate donor kinetics as well as differences in dCTP inhibition between human and mouse dCK may be important determinants of the *in vivo* enzyme properties. We have identified differences between the purified enzymes that, however, do not rule out other modifications of the enzymes in their natural environment.

Acknowledgements—This work was supported by grants from the Medical Faculty of the Karolinska Institute, the Swedish Society for Medical Research, the Biomedical Research Programme and the Human and Capital Mobility Programme of the European Community. The authors thank Birgitta Wallström for her valuable technical assistance.

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