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NUCLEOSIDE TRIPHOSPHATE DONORS FOR NUCLEOSIDE KINASES: DONOR PROPERTIES OF UTP WITH HUMAN DEOXYCYTIDINE KINASE

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SUMMARY The reported higher efficiency of UTP, relative to ATP, as phosphate donor for deoxycytidine kinase (dCK), has been extended and found to apply to both dCyd and dAdo as acceptors. UTP as phosphate donor was shown to follow strictly Michaelis kinetics, with K_m = 1 μM, in striking contrast to ATP, which exhibits marked negative cooperativity (Hill coef. = 0.7) with a several-fold higher K_m^{app} = 15 μM. Phosphate transfer was followed directly with use of mixtures of [γ-^{32}P]ATP and cold UTP as donors, or with ^{3}H-labeled acceptors and cold donors. With equimolar concentrations of ATP and UTP (50 μM or 1 mM each), and dCyd or dAdo as acceptor, only minimal phosphate transfer occurred from ATP (3-10%). With a 6:1 ratio of ATP:UTP, hence exceeding the intracellular ratio, phosphate transfer from ATP increased, but still did not exceed 25-40% with either dCyd or dAdo as acceptor. Moreover, relative ATP transfer is dependent on the dCyd concentration. We conclude that the major intracellular phosphate donor for dCK is not ATP, but UTP. Preliminary data for human thymidine kinases (TK1 and TK2) exhibit quite different behaviour. The foregoing, together with literature data, are highly relevant to *in vitro* studies on the properties of the nucleoside kinases, and to the design of chemotherapeutically active nucleoside analogues.

Although widely and implicitly assumed that ATP is the universal intracellular phosphate donor in kinase reactions, there are numerous exceptions in *in vitro* studies. For example, with protein kinases, GTP may effectively substitute for ATP with protein kinase CK-II, but not CK-I [1]. Attention has been drawn to the ability of other NTPs to function as phosphate donors [1,2].

In the field of nucleoside kinases, it has long been known that a variety of NTPs and NTP analogues may be as effective, or even more so, than ATP (see [1,2] and references cited). It was

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Abbreviations: NTP, nucleoside 5'-triphosphate; dCK, deoxycytidine kinase; dGK, deoxyguanosine kinase; TK1, cytosolic thymidine kinase; TK2, mitochondrial thymidine kinase.

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long ago shown by Ives and co-workers that UTP is more effective than ATP as a phosphate donor with dCK [3], and that UTP, CTP and dTTP were more effective donors than ATP at physiological pH for dGK [4]. Nonetheless, it is tacitly assumed that the higher concentration of ATP, relative to other NTPs, favours ATP as the effective *in vivo* donor. However, White and Capizzi [5] showed that the kinetics of phosphorylation of araC by dCK *in vivo* is more consistent with *in vitro* results obtained with UTP as sole donor. In a subsequent report by Shewach et al. [6] on the dCK from cultured MOLT-4 T lymphoblasts, UTP was also more efficient than ATP as phosphate donor; and phosphorylation of dCyd was even further enhanced in the presence of a mixture of MOLT-4 cellular concentrations of the eight endogenous NTPs. Furthermore, under these conditions, the feed-back inhibition by dCTP, commonly observed *in vitro*, is virtually liquidated by the UTP in the NTP mixture used as donor.

The present investigation is an extension of the foregoing, with a view to gaining further insight into the nature of the intracellular phosphate donor for dCK. This enzyme has been extensively investigated, not only in relation to its normal physiological functions, but also because of its key role as the rate-limiting activation step for a number of nucleoside antitumour and antiviral agents [2,5].

MATERIALS AND METHODS

Materials. [5-^{3}H]dCyd (18.2 Ci/mmol), [6-^{3}H]dThd (30 Ci/mmol) and [γ-^{32}P]ATP (>5000 Ci/mmol) were purchased from the Radiochemical Centre, Amersham. [2,8-^{3}H]dAdo (44 Ci/mmol) was from Moraveck Biochemicals, Inc. (Brea, California). Unlabeled nucleosides, ATP and bovine serum albumin (fraction V) were obtained from Sigma Chemical Co. UTP from Calbiochem (La Jolla, California) was additionally purified as described below.

UTP Purification It has been reported [5] that UDP is at least a 10-fold more potent inhibitor of dCK activity with ATP as a phosphate donor (K_i=4 μM) than with UTP. Hence the presence of UDP contamination significantly inhibits ATP activity without affecting UTP activity - a most undesirable effect when studying ATP/UTP donor mixtures (see Results). Even the best commercially available UTP preparations contain about 3% contaminants, principally UDP, the level of which increases during storage, even at -20 °C. It was therefore essential to purify UTP, as follows: 80 mg UTP (Calbiochem, nominal purity 97%, actually 90% due to storage) was dissolved in 20 ml water and applied to a 3.5 × 50 cm column of DEAE -Sephadex (A-25, HCO_3^- form). The column was washed with water and then eluted with a 0 - 1 M linear gradient of TEAB, pH 7.5 (1.5 l). Pure UTP eluted as a sharp peak at about 0.8 M TEAB, well separated from the preceding UDP peak. UTP peak fractions were pooled, desalted and converted to the corresponding Na^+ salt by ion exchange. Final purity of UTP (≥99%) was checked by HPLC on a reverse phase Supelco C18 column with an ion-pair solvent system [7].

Enzymes. Human leukemic spleen was the source of dCK [8], as well as TK1 and TK2 [9], all purified to homogenity. The TK1 preparation was the so-called low affinity form [10].

Enzyme Assays. dCK activity was first followed by a radiochemical assay procedure with ^{3}H-labeled acceptors [11] with some modifications, including a lower total reaction volume: 50 µl instead of 200 µl; lower level of enzyme (7-15 ng for dCyd assays, about 50 ng for dAdo) and smaller aliquots spotted on DEAE discs (10 µl instead of 40 µl). Liquid scintillation counting was performed with 5 ml Bray scintillator. Initial reaction velocities were determined using linear regression analysis (the reaction course was linear during 30 min.), and kinetic parameters calculated by means of non-linear regression analysis. TK activity was followed with ^{3}H-labeled dThd in the same way [9].

dCK and TK activities were also monitored with the use of [γ-^{32}P]ATP [12]. Reaction conditions were as above, with replacement of tritium-labeled nucleosides by nonlabeled ones. Phosphate donors were ATP or ATP/UTP mixtures with addition of 2-12 μCi [γ-^{32}P]ATP per reaction. Background reactions were also performed, with the same components, but without phosphate acceptor. The reaction was terminated after 30 min by boiling for one min and 6-10 μl of the reaction mixture applied to a PEI-cellulose thin layer plate (Merck). Chromatography was performed twice in the same direction for about 10 hours (each), using isobutyric acid : conc. NH_4OH . H_2O (66:1:33, v/v) as the mobile phase. Reaction products were detected by autoradiography, the spots excised and eluted with 0.5 ml of 0.2 M KCl/0.1 M HCl (1:1, v/v) and quantitated by liquid scintillation. Fragments of the same R_f and area as the product were excised from the background and subtracted. Products formed did not exceed 2 μM which, in the case of UDP, was too low to result in significant inhibition of the reaction (see above).

Both assay procedures were also used simultaneously to measure phosphate uptake from ATP (and UTP) when mixtures of phosphate donors were used (such measurements could also be performed with [γ-^{32}P]UTP, but this was not available). The ^{3}H assays yield the total amount of product formed at a given ATP/UTP ratio, while the ^{32}P assay counts only product formed from ATP, so the lacking amount of product comes from UTP. More precisely, and for better accuracy, four reactions were performed for each ATP/UTP ratio: two ^{3}H assays: one with the ATP/UTP mixture and a second with ATP alone, and two assays under the same conditions with ^{32}P-labeled ATP. The concentration of ATP alone was in the same range as that of the donor mixture. Relative phosphate uptake from ATP (and UTP) was calculated as follows:

$$\% \text{ product from ATP} = \frac{^{32}\text{P product (ATP / UTP)}}{^{32}\text{P product (ATP)}} \bullet \frac{^{3}\text{H product (ATP)}}{^{3}\text{H product (ATP / UTP)}} \bullet 100\%$$

$$\% \text{ product from UTP} = 100\% - \% \text{ product from ATP}$$

One may, of course, determine the relative substrate activity of ATP simply on the basis of ^{3}H and ^{32}P assays with an ATP/UTP mixture. However, the foregoing procedure possesses the advantage that it removes uncertainties in those instances where possible radioactive contamination of preparations of labeled compounds is suspected.

RESULTS

It was first established that the human dCK employed with dCyd as acceptor displayed a decrease in the kinetic parameters K_m and V_{max} when the ATP donor was replaced by UTP [*cf.* 5,6]. The effect observed was virtually identical with that for the enzyme from MOLT-4 cells [6]. Replacement of the routinely employed 1 mM ATP by 1mM UTP led to a 3.5-fold decrease in V_{max} (2.2-fold with the MOLT-4 enzyme) and a 5-fold decrease in K_m (3.6-fold with MOLT-4), giving a 1.5-1.6-fold higher V_{max}/K_m value with UTP as donor for both enzymes. A similar pattern was obtained with the other natural acceptor substrate of dCK, dAdo, although with a distinctly smaller V_{max}/K_m increment (Table 1).

Furthermore, when dCyd was employed as acceptor with a 1:1 mixture of ATP and UTP as donor (each 0.5 mM), kinetic parameters for phosphorylation of dCyd were virtually identical with those obtained by use of 1mM UTP, as previously noted with araC as acceptor [5], underlining the superiority of UTP as donor in this system. This prompted us to examine in more detail the behaviour of UTP. With 1 μM dCyd as acceptor, kinetic parameters were determined

TABLE 1. Kinetic parameters for phosphorylation of dCyd and dAdo with 1 mM ATP or UTP as phosphate donors

Phosphate acceptor	Phosphate donor	Kinetic parameters [a]		
		K_m (μM)	V_{max}	V_{max}/K_m
dCyd	ATP	0.8±0.1	100±10 [b]	125±20
	UTP	0.15±0.02	28.0±1.3	187±25
dAdo	ATP	35±4	315±15	9±1
	UTP	9±1	91±3	11±1

[a] dCK exhibits negative cooperativity with both phosphate acceptors, described by a model with two K_m and V_{max} values, one for the lower, the other for the higher acceptor concentations [11,16]. These parameters are for the lower concentration range ($\leq 4 \cdot K_m$)

[b] The V_{max} value for dCyd (27.9 nmol/min/mg) with ATP as donor is taken as 100

for UTP over the range 0.2-100 μM, bearing in mind that ATP exhibits negative cooperativity [8,13]. The K_m value obtained (1.0 ± 0.1 μM) is in accord with those reported by Cheng et al. [14] and White and Capizzi [5]. We further found our data in accord with a Hill coefficient n = 1.0 (± 0.1), consistent with Michaelis kinetics. This is in striking contrast to ATP, for which we found a K_m^{app} = 15 (± 0.1) μM , and a Hill coefficient n = 0.7 (± 0.1), and underlines, albeit indirectly, the potential key role of UTP in the phosphotransfer reaction, as in previous reports [5,6]

The extent of phosphate transfer, and the source of the phosphate group, was then examined with the use of mixtures of the two donors, UTP and [γ-^{32}P]ATP. Three mixtures of donors were employed. Two of these were equimolar, 50 μM of each and 1 mM of each (the donor concentration widely employed in *in vitro* assays); and 300 μM ATP : 50 μM UTP, approximately the average intracellular ratio of these NTPs, ~ 3mM : 0.5 mM [15].Our use of 10-fold lower concentrations was dictated by technical considerations, permitting more accurate assays. Reactions were conducted with both natural acceptors, dCyd and dAdo. Since the enzyme kinetics [11,16] and ligand binding [17] with dCK exhibit negative cooperativity for both of these, which may be approximately described by two Michaelis reactions with K_m and V_{max} values for low and high concentrations of the acceptor (K_m and V_{max} being higher for the larger acceptor concentration), two concentration of each acceptor, slightly higher than the individual K_m values, were employed with each mixture of donors. The results (see Table 2) are expressed as the ratio of the amount of product from ATP to total product from both donors.

For equimolar concentrations of ATP and UTP, very low phosphate transfer occurred from ATP (3-10%) to both phosphate acceptors at low and high concentrations of the latter. With a 6:1 ratio of ATP:UTP, phosphate transfer from ATP increased, but did not exceed

TABLE 2. Relative phosphate uptake (%) from the ATP component of an ATP/UTP mixture catalyzed by dCK shown as a percentage of total transfer from the mixtures of both phosphate donors

Phosphate acceptors (μM)		Phosphate donors, ATP : UTP (% of phosphate uptake from ATP) [a]		
		50μM:50μM	1mM:1mM	300μM:50μM
dCyd	2.5	5	4	29
	100	10	8	41
dAdo	100	3	8	34
	1000	3	8	23

[a] Relative accuracy (15%), estimated from two independent determinations.

25-40% for both acceptors. Relative phosphate transfer is increased at a higher concentration of dCyd, most likely due to the negative cooperativity induced by this acceptor. With equimolar mixtures of the two donors, the activity of ATP at the lower concentration is only one-half that at the higher dCyd concentration. With the 6:1 ATP/UTP mixture, the activity of the ATP component is reduced by 30 % at the lower dCyd concentration. The absence of this effect with dAdo (Table 2) is probably due to its known negative cooperativity pronounced only at very high concentrations. To this must be added the known negative cooperativity of ATP, so that its relative activity is probably dependent on its concentration in the equimolar mixtures. This effect is evident with dAdo, where the activity of ATP increases from 3 % to 8 % when its concentration is raised from 0.05 to 1 mM. It is not clear why, in the case of dCyd, this is unchanged (Table 2).

The foregoing points to the desirability of examining the effects of UTP with other nucleoside kinases. The availability of highly purified preparations of cytoplasmic TK1 and mitochondrial TK2 prompted us to examine the donor properties of UTP with these enzymes. UTP alone was a moderate donor with TK2, and somewhat poorer with TK1, relative to ATP. However, with a [γ-^{32}P]ATP:UTP (50 μM: 50μM) mixture as donor, there was no detectable phosphate transfer from UTP to Thd (at 1 μM concentration) with TK2, whereas with TK1 only 12 % phosphate transfer to Thd (15 μM) originated from UTP. These preliminary findings are being extended to other potential NTP donors and mixtures of these.

DISCUSSION

The results of White & Capizzi [5] and Shewach et al. [6] furnished indirect evidence for UTP as the major phosphate donor with dCK. The present findings, based on the use of

UTP/[γ-^{32}P]ATP mixtures as phosphate donor, with identification of phosphorylated acceptors and the source of the phosphate, remove any doubts that may have existed on this score. From Table 2 it is clear that the extent of phosphate transfer from the ATP component of an ATP/UTP mixture is strikingly low; and, with equimolar ratios of ATP and UTP, does not exceed 10 %. Even with an ATP/UTP ratio of 6:1, which is somewhat higher than the intracellular ratio for many cells, particularly tumour cells [6,15], phosphate transfer from ATP barely attains a level of 40 %. Note also, from Table 2, that the superiority of UTP as donor is retained over a broad range of donor and acceptor concentrations, albeit changes of an order of magnitude affect relative donor efficiencies to only a small extent.

Our findings with dCK, although limited to use of only two acceptors, suggest that replacement of one NTP donor by another probably does not alter the relative efficiency of phosphate acceptors. This is, in fact, confirmed by a report (which appeared during completion of this manuscript) that five acceptor substrates of dCK exhibited relative V_{max}/K_m values in the same order with either ATP alone or UTP alone as donor [18].

The situation with inhibitors may be more complex. Although replacement of one donor by another may not affect the relative order of inhibitor properties, their K_i values may differ significantly, as found for dCK with UDP and dCTP [5,6], both of which are much more potent inhibitors with ATP than with UTP. Moreover, the inhibitory effects of these two *in vivo* could conceivably shift phosphate uptake by an acceptor to an even greater extent with UTP than shown in Table 2.

Extension of the present investigation to other potential NTP donors and acceptors, as well as to other kinases, is clearly necessary, and our preliminary findings with TK1 and TK2 are an initial step in this direction. Particularly interesting in this context should be dGK, for which UTP, dTTP and CTP are much better donors than ATP at physiological pH [4].

Finally, it should be recalled that many nucleoside analogues with antitumour and antiviral activities are dependent for their activity on prior intracellular phosphorylation by nucleoside kinases, in numerous instances by dCK. But, as pointed out by White and Capizzi [5], the interaction of these analogues with dCK has hitherto been limited to use of ATP as donor, with results which, in the case of araC, do not fit the kinetics of phosphorylation in human leukemic cells [5] or in araC-resistant leukemic cells [19]. The foregoing, and a recent report by Wang et al. [20] demonstrate that the *in vitro* substrate/inhibitor properties of nucleoside analogues must be conducted under conditions which simulate as close by as possible those prevailing in cellular systems. Appropriate attention to this point should facilitate the design of more effective chemotherapeutic nucleoside analogues.

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