Kinetic Analysis of Human Deoxycytidine Kinase with the True Phosphate Donor Uridine Triphosphate†

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Received January 10, 1997; Revised Manuscript Received March 20, 1997⊗

ABSTRACT: Deoxycytidine kinase is the rate-limiting process in the activation for several clinically important antitumor agents. Previous studies have focused on deoxycytidine (dCyd) and adenosine triphosphate (ATP) as substrates for this enzyme. In view of recent data indicating that uridine triphosphate (UTP) is the physiologic phosphate donor for this enzyme, a study of the kinetic properties of dCyd kinase with dCyd and UTP was undertaken. The results presented here demonstrate that UTP and ATP produce kinetically distinguishable differences in nucleoside phosphorylation by dCyd kinase. At high dCyd concentrations, dCyd kinase exhibited substrate activation with ATP. In contrast, in the presence of UTP, substrate inhibition was observed at concentrations of dCyd greater than 3 μM. Inhibition by dCyd was noncompetitive with respect to UTP and could not be reversed by a 200-fold increase in UTP concentration, indicating that the inhibition was not due to dCyd binding at the nucleotide binding site. The kinetic mechanism for dCyd kinase was determined with dCyd and UTP as substrates. UTP was the preferred phosphate donor with a true K_m value of 1 μM compared to 54 μM with ATP, resulting in a 50-fold greater substrate efficiency for UTP. Although the double-reciprocal plots with UTP produced parallel lines, initial velocity plots with other phosphate donors and product inhibition studies indicated that dCyd kinase formed a ternary complex with its substrates. The parallel lines with UTP were apparently due to a low dissociation constant for UTP, which was calculated as more than 13-fold lower than its K_m value. Analysis of product inhibition studies indicated that dCyd kinase followed an ordered A-B random P-Q reaction sequence, with UTP as the first substrate to bind. In contrast, previous results demonstrated a random bi-bi sequence for dCyd kinase in the presence of ATP. The combined results indicate that the enzyme can follow a random bi-bi reaction sequence, but with UTP as the phosphate donor, the addition of nucleotide prior to dCyd is strongly preferred. The noncompetitive substrate inhibition, which was independent of UTP concentration, indicates that high concentrations of dCyd promote addition of the nucleoside prior to UTP, resulting in a lower velocity.

Deoxycytidine kinase (E.C. 2.7.1.74) has evoked interest because of its unique role in the initial phosphorylation of several nucleoside analog antitumor and antiviral agents to their therapeutic 5′-triphosphate derivatives (Krenitsky et al., 1976; Arner & Eriksson, 1995). While dCyd[1] kinase has highest affinity for dCyd analogs, such as araC, dFdCyd, and dideoxycytidine, this enzyme can also phosphorylate several purine analogs, including fludarabine, chlorodeoxyadenosine, arabinosylguanine, and 2-chloroadenine-2′-fluoroarabinoside (Plunkett & Saunders, 1991; Arner & Eriksson, 1995; Heinemann et al., 1988; Shewach et al., 1992; 1993; Saven et al., 1993; Parker et al., 1991; Xie & Plunkett, 1995). The central role of dCyd kinase in the activation of these clinically important compounds has prompted evaluation of the biochemical properties and substrate specificity for this salvage pathway enzyme (Krenitsky et al., 1976; Sarup & Fridland, 1987; Bohman & Eriksson, 1988; Datta et al., 1989a; Kim & Ives, 1989).

In addition to its broad nucleoside substrate specificity, studies with the purified enzyme have shown that dCyd kinase can accept a variety of 5′-triphosphates as phosphate donors (Cheng et al., 1977; Datta et al., 1989a; White & Capizzi, 1991; Shewach et al., 1992). Of the endogenous cellular nucleotides, only dCTP does not function as a phosphate donor but instead is an inhibitor of dCyd kinase (Sarup et al., 1989; Datta et al., 1989a; White & Capizzi, 1991; Shewach et al., 1992). Since the velocity of the dCyd kinase reaction was highest with ATP, studies of nucleoside affinity for dCyd kinase have utilized ATP as the phosphate donor (Datta et al., 1989a; Eriksson et al., 1991). Under these conditions, nonlinear initial velocity kinetics were observed at high concentrations of pyrimidine and purine nucleosides, indicative of substrate activation (Sarup & Fridland, 1987). In view of the fact that dCyd kinase is composed of two identical subunits (Bohman & Eriksson, 1988; Sarup & Fridland, 1987; Datta et al., 1989a; Chottiner et al., 1991), these data suggest that there are two catalytic sites on the enzyme that cooperate to produce substrate activation.

More recent studies have demonstrated that UTP and not ATP is the physiologic phosphate donor (White & Capizzi, 1991; White & Hines, 1987; Shewach et al., 1992; Krawiec et al., 1996). Data from this and other laboratories have demonstrated that, compared to results with ATP as the

† This work was supported in part by U.S. Public Health Service Grant CA 46452 and American Cancer Society Grant DHP-85D.

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⊗ Abstract published in *Advance ACS Abstracts,* May 15, 1997.

[1] Abbreviations: dCyd, deoxycytidine; araC, 1-(β-D-arabinofuranosyl)cytosine; dFdCyd, 2′,2′-difluoro-2′-deoxycytidine.

S0006-2960(97)00059-7 CCC: $14.00

phosphate donor, nucleoside substrate affinity for dCyd kinase in the presence of UTP resulted in consistently lower K_m values for most substrates (Shewach et al., 1992; White & Capizzi, 1991; Krawiec et al., 1996). While V_{max} values were also lower, the overall efficiency of the reaction was highest with UTP as the phosphate donor, based on relative V_{max}/K_m values (Shewach et al., 1992; Krawiec et al., 1996). Interestingly, the nonphysiologic (−) enantiomers of 2′,3′-dideoxy-3′-thiacytidine and its 5-fluoro analog showed the opposite effects on K_m and V_{max} values: both were higher with ATP compared to UTP as the phosphate donor, again resulting in a kinetic advantage for UTP (Shewach et al., 1993). These data clearly establish the importance of UTP in the dCyd kinase reaction.

Despite the evidence that UTP is the preferred phosphate donor, detailed kinetic studies of dCyd kinase with dCyd and UTP as substrates are lacking. In addition, there are conflicting reports in the literature concerning the kinetic behavior of dCyd kinase at high nucleoside concentrations. One report indicated that araC phosphorylation decreased at high concentrations (White & Capizzi, 1991), whereas a recent brief report indicated that typical Michaelis−Menten kinetics are observed with high concentrations of dCyd (Krawiec et al., 1996). This is an important point to resolve since chemotherapeutic agents that are substrates for dCyd kinase, such as araC, are commonly administered at high concentrations (Heinemann & Jehn, 1990). To address this issue, we have compared the kinetics of phosphorylation over a broad range of dCyd concentrations with either ATP or UTP as phosphate donor. In addition, we have analyzed the kinetic mechanism for dCyd and the true phosphate donor, UTP, since previous studies of the reaction sequence for this enzyme have utilized ATP exclusively as the phosphate donor (Kim & Ives, 1989; Datta et al., 1989b). The results presented here demonstrate that both the kinetic behavior at high dCyd concentrations and the preferred reaction sequence differed with UTP compared to ATP as the phosphate donor. A model is presented that integrates the effects at high dCyd concentrations with the proposed reaction sequence for dCyd kinase. These results have important implications for the optimal clinical administration of chemotherapeutic agents such as araC and dFdCyd, which are dCyd kinase substrates.

MATERIALS AND METHODS

Materials. [5-^{3}H]dCyd was purchased from Moravek Biochemicals, and its purity was >97% according to reverse-phase HPLC analysis. [γ-^{32}P]UTP and [γ-^{32}P]ATP were obtained from ICN Radiochemicals, Irvine, CA, and were >99% pure by strong anion-exchange HPLC analysis. Thin-layer chromatography silica plates and DE-81 filter discs were purchased from Whatman Labsales, Hillsboro, OR.

Deoxycytidine Kinase Assay Using [^{3}H]dCyd. dCyd kinase was isolated from MOLT-4 T lymphoblasts to greater than 90% purity by a procedure described previously (Shewach et al., 1992). Purified dCyd kinase was desalted once or twice to remove UTP and dCyd that remained in the preparation from the purification procedure. Desalting was accomplished on a Sephadex G-25 column (Isolab, Inc.) as described (Shewach et al., 1992) and then the enzyme was diluted before assay. The final enzyme preparation after single desalting contained <0.025 μM dCyd and <0.5 μM UTP. Double-desalted enzyme used for true K_m studies contained <0.5 nM dCyd and <100 nM UTP. The complete reaction mixture contained variable concentrations of tritiated nucleoside substrate, nucleoside 5′-triphosphate and $MgCl_2$ at equimolar concentrations, 50 mM imidazole, pH 7.4, 25 mM dithiothreitol, 5% glycerol, 100 mM KCl, and 1 mg/mL BSA in a final volume of 30 μL. Kinetics were similar when a constant excess of $MgCl_2$ ($\leq$200 μM) was added to the reaction. The reaction mixture was incubated in a water bath at 37 °C for 10 min, followed by inactivation at 85 °C for 1 min. Aliquots of the reaction mixture were pipetted onto DE-81 filter discs, which were washed with ammonium formate, and radioactivity was quantitated as described (Shewach et al., 1992). In all assays, less than 10% of the substrate was converted to product. Phosphorylation of dCyd was linear at all concentrations tested for at least 50 min. All assays were performed in duplicate, and each experiment was performed at least twice. K_m and V_{max} values were determined with the aid of a computer program that uses a linear regression method with weighting of velocities according to proportional error (Wilkinson, 1961). K_i values were calculated from replots of inhibitor concentration vs slope or intercept of the double-reciprocal plots using linear least-squares regression analysis to estimate the best-fit line describing the data points.

Phosphorylation of UDP to UTP with Pyruvate Kinase. Pyruvate kinase was added to the dCyd kinase assay to test whether accumulation of high concentrations of UDP was inhibitory to dCyd kinase. Assay conditions were as described above for dCyd kinase with the addition of 1 unit of pyruvate kinase and 5 mM phosphoenolpyruvate. UDP and UTP were separated and quantitated by strong anion-exchange HPLC analysis as described previously (Shewach et al., 1992). This analysis demonstrated that there was <3% contaminating UDP in the UTP preparations. Pyruvate kinase under the conditions used converted on average >90% of the UDP to UTP during the 10 min incubation.

Deoxycytidine Kinase Assay Using ^{32}P-Labeled Nucleotides. Deoxycytidine kinase was incubated essentially as described above with the following changes. [5-^{3}H]Deoxycytidine was used at a fixed concentration (5 μM) with variable concentrations of either [γ-^{32}P]UTP (1−10 μM) or [γ-^{32}P]ATP (20−200 μM). Unlabeled ATP was added as an inhibitor of [γ-^{32}P]UTP utilization, and in the complementary study UTP was included as an inhibitor of [γ-^{32}P]-ATP utilization. The reaction mixture was incubated for 10 min at 37 °C, followed by inactivation for 1 min at 85 °C. Aliquots of the reactions mixture were pipetted onto polyester-backed, 250 μm thin-layer chromatography silica plates that were prespotted with unlabeled dCMP and either UTP or ATP. Radioactive dCMP product was separated from other products and substrates by developing the plates in a chamber containing ammonium hydroxide−2-propanol−dH_2O (30:56:14). Migration of dCMP was identified by UV absorbance (254 nm) of the unlabeled standard, the area was cut out and placed into scintillation vials, and the radioactivity was eluted with dH_2O for 10 min prior to the addition of 10 mL of Biosafe liquid scintillant. The amounts of ^{3}H- and ^{32}P-labeled dCMP were quantitated in a Beckman LS 6000SC scintillation spectrometer. Velocity of the dCyd kinase reaction with ^{32}P-labeled nucleotide was calculated from the amount of [^{32}P]dCMP.

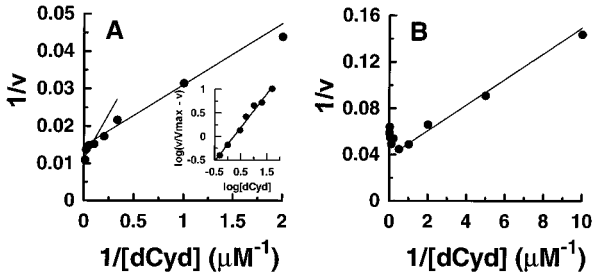


FIGURE 1: Pattern of dCyd phosphorylation with ATP or UTP as the phosphate donor. (A) [^{3}H]dCyd (0.5−100 μM) and 2 mM ATP. Graph represents a typical experiment. (Inset) Hill plot of data from panel A. (B) [^{3}H]dCyd (0.2−100 μM) and 2 mM UTP as the phosphate donor.

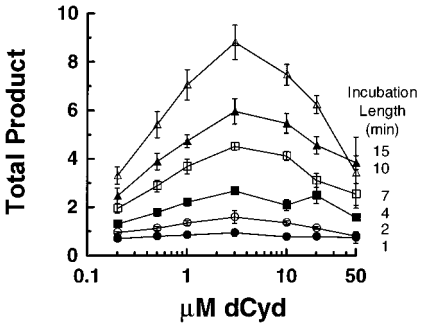


FIGURE 2: Total product formed with time. Deoxycytidine kinase was incubated with [^{3}H]dCyd (0.2−50 μM) and 10 μM UTP for 1−15 min. Total product represents total amount of [^{3}H]dCMP formed in the corresponding incubation period. Data points represent the average ± SD of duplicate determinations.

RESULTS

Previously it has been reported that dCyd kinase exhibited enzyme activation with purine and pyrimidine substrates at high concentrations when ATP was utilized as the phosphate donor (Sarup & Fridland, 1987), whereas there are conflicting reports on the kinetic behavior of dCyd kinase at high dCyd concentrations with UTP as the phosphate donor (White & Capizzi, 1991; Krawiec et al., 1996). To clarify whether high concentrations of dCyd produce different kinetic results with ATP relative to UTP, we compared the phosphorylation by dCyd kinase over a broad range of dCyd concentrations with either 2 mM ATP or UTP as the phosphate donor. Similar to a previous report (Sarup & Fridland, 1987), when ATP was used as the phosphate donor, the velocity of dCyd phosphorylation increased as the dCyd concentration increased up to 100 μM, characteristic of substrate activation (Figure 1A). Two apparent K_m values were calculated in linear regions of the double-reciprocal plot from 0.5 to 5 μM dCyd (K_{m1} = 1.1 μM) and from 10 to 100 μM dCyd (K_{m2} = 4.1 μM). A Hill plot of these data indicated negative cooperativity with a value of n = 0.7 (Figure 1A, inset). In marked contrast, when UTP was utilized as the phosphate donor, no activation occurred but instead a decrease in phosphorylation was observed at dCyd concentrations above 10 μM (Figure 1B). An apparent K_m value of 0.2 was calculated between 0.2 and 3 μM dCyd. Additional studies demonstrated that these kinetic differences noted in Figure 1 were not due to differential aggregation of subunits in the presence of either ATP or UTP. Elution of native enzyme from a Superose-12 column in the presence of 2 mM ATP or UTP yielded a molecular mass of 60 kDa, indicating that in the presence of either nucleotide dCyd kinase is a dimer as previously reported (Datta et al., 1989a). Further studies demonstrated that the observed inhibition was independent of the UTP concentration since experiments with 100 or 10 μM UTP produced results similar to those presented in Figure 1B (data not shown).

To understand the mechanism by which dCyd kinase was inhibited at high nucleoside substrate concentrations, the end products of the reaction were evaluated for inhibition of dCyd phosphorylation at a saturating concentration of UTP (2 mM). Inhibition studies demonstrated that dCMP was a weak, mixed inhibitor of the enzyme with K_{ii} and K_{is} values of 6.9 and 19.4 mM, respectively. Therefore, concentrations of dCMP generated during the dCyd kinase assay were too low to inhibit the enzyme significantly. UDP was a more potent, uncompetitive inhibitor with a K_i value of 735 μM. Although this is a relatively high K_i value, the presence of contaminating UDP in the UTP preparation (~3%) plus UDP generated during the assay may result in enzyme inhibition. To test this hypothesis, studies similar to those described in Figure 1B were performed in the presence of pyruvate kinase to rapidly convert any UDP generated to UTP. Analysis of the reaction products by HPLC demonstrated that the pyruvate kinase converted >90% of the UDP to UTP (data not shown). While the addition of pyruvate kinase increased the velocity slightly (average 21.0% ± 3.4%; range 4.0−34.8%) at dCyd concentrations of at least 5 μM, it could not prevent the inhibition completely (data not shown). Furthermore, the total velocity decreased at dCyd concentrations greater than 10 μM whether or not pyruvate kinase was present. Thus, production of UDP during the reaction may have decreased the velocity somewhat but could not account fully for the observed inhibition at high dCyd concentrations.

To further characterize the inhibition of dCyd kinase, the effect of incubation length on the amount of dCMP formed during the reaction was examined (Figure 2). If a product generated during the course of the reaction was responsible for the inhibition, then the formation of dCMP should decrease with increasing incubation time. In these studies, the total amount of dCMP increased linearly over the 1−15 min incubation periods at all dCyd concentrations. However, at concentrations of dCyd greater than 3 μM, the amount of dCMP produced at all time points was less than at lower dCyd concentrations. The results demonstrate that enzyme inhibition is dependent upon dCyd concentration and not length of incubation. Thus these data confirm that end-product inhibition cannot account for the kinetics presented in Figure 1B.

The results in Figure 2 suggested that dCyd was directly causing enzyme inhibition at high concentrations. To determine the nature of this inhibition, studies employing increasing concentrations of dCyd versus variable UTP concentrations were performed. These studies indicated that dCyd produced noncompetitive substrate inhibition with respect to UTP (Figure 3). It should be noted that the substrate inhibition was only partial since a measurable velocity was obtained at all concentrations of dCyd tested (Figures 2 and 3). Based on the noncompetitive nature of this inhibition, the data indicate that dCyd does not bind to the nucleotide binding site to cause the inhibition.

Since the kinetics of phosphorylation of dCyd were markedly different in the presence of UTP compared to ATP, initial velocity experiments were performed to determine the

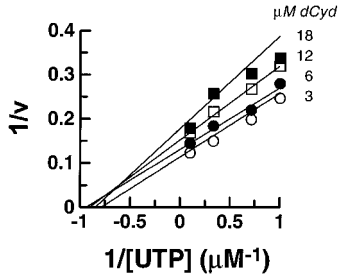


FIGURE 3: Inhibition of phosphorylation at high dCyd concentrations with constant UTP. Deoxycytidine kinase was incubated with [^{3}H]dCyd as indicated and 10 μM UTP. Concentrations of dCyd below 3 μM did not inhibit the enzyme.

Table 1: Kinetic Values for dCyd with Various Nucleotide Substrates[a]

	dCyd		nucleotide		
nucleotide substrate	true K_m (μM)	relative V_{max}/K_m	true K_m (μM)	relative V_{max}/K_m	K_{ia} (μM)
UTP	0.5	1.00	1.2	1.00	0.09
ATP	1.0	0.44	54	0.02	41
GTP	0.9	0.65	33	0.05	12
CTP	1.3	0.10	9.0	0.03	10
dTTP	1.0	0.58	2.2	0.57	0.7

[a] True K_m studies were performed using at least three different concentrations of dCyd and at least four different concentrations of nucleotide. Double-reciprocal plots of the data yielded patterns of lines that intersected at a common point, with the exception of the UTP data, which yielded parallel lines (Figure 4). Replots of 1/[substrate] vs slope or intercept were linear.

true kinetic constants with dCyd and UTP or ATP as substrates. In the presence of UTP the true K_m for dCyd was 0.5 μM, compared to 1.0 μM in the presence of ATP (Table 1). The V_{max}/K_m measure of substrate efficiency indicated a preference for UTP by a factor of 2.3-fold. Larger differences were found in the relative values for the phosphate donors, in which UTP was strongly preferred over ATP with respective K_m values of 1.2 and 54 μM, with a 50-fold greater efficiency for UTP as indicated by the V_{max}/K_m value (Table 1). Since dCyd kinase can accept a variety of endogenous nucleoside 5′-triphosphates as phosphate donors, we also compared the true K_m values for the nucleotides GTP, CTP, and dTTP (Table 1). These studies demonstrated that, of all nucleotides tested, UTP is the preferred phosphate donor for the dCyd kinase reaction, with dTTP as the next best nucleotide substrate. On the basis of relative values for K_m and substrate efficiency, ATP was the poorest of all phosphate donors tested.

Interestingly, the initial velocity plots when both dCyd and the phosphate donor were varied showed large differences depending upon the nucleotide used. As illustrated in Figure 4, when dCyd and UTP were varied, the double-reciprocal plots yielded a series of parallel lines, in contrast to the converging pattern of lines found when dCyd and ATP were varied (Datta et al., 1989b). While the pattern displayed in Figure 4 is suggestive of a two-step transfer (ping pong) mechanism (Segel, 1975), it seemed highly unlikely that dCyd kinase would form a binary substrate−enzyme complex with UTP, in contrast to a ternary complex in the presence of ATP. Initial velocity kinetics performed with the other nucleotide phosphate donors shown in Table 1 also produced a series of converging lines (data not shown). These data strongly suggest that the kinetic mechanism for dCyd kinase

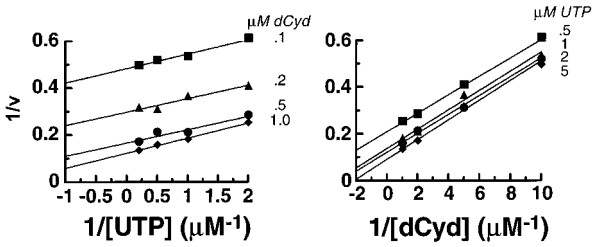


FIGURE 4: Determination of true kinetic constants for dCyd and phosphate donors. [^{3}H]dCyd was varied from 0.2 to 1.2 μM, and UTP, from 1 to 10 μM. Similar plots were obtained when UTP was varied from 0.1 to 10 μM.

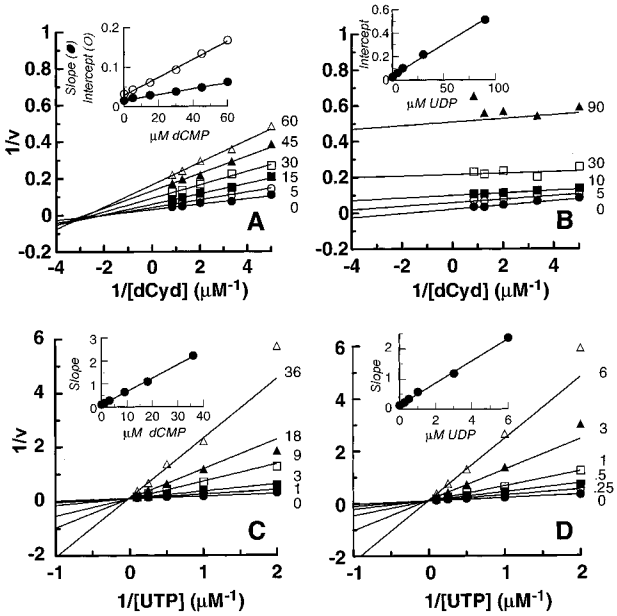


FIGURE 5: Product inhibition studies with dCyd and UTP as substrates for dCyd kinase. (A) Inhibition of dCyd phosphorylation by dCMP; (B) inhibition of dCyd phosphorylation by UDP; (C) inhibition of UTP utilization by dCMP; (D) inhibition of UTP utilization by UDP. In all panels, UTP was present at a saturating concentration of 10 μM.

Table 2: Kinetic Constants for Product Inhibitors of dCyd Kinase

kinetic constant	value (μM)	varied substrate(s)	concn of fixed substrate	pattern of inhibition[a]
K_i UDP	0.4	UTP	2 μM dCyd[b]	competitive
K_i dCMP	1.5	UTP	2 μM dCyd[b]	competitive
K_i UDP	6.9	dCyd	10 μM UTP[c]	uncompetitive
K_i dCMP	22	dCyd	10 μM UTP[c]	noncompetitive

[a] Pattern of inhibition describing data from double-reciprocal plots. [b] Four times true K_m value; higher concentrations of dCyd cause inhibition. [c] Saturating concentration (10 times true K_m value).

involves formation of a ternary complex of enzyme with both substrates.

Product inhibition studies were performed to define the dCyd kinase reaction sequence when dCyd and UTP were used as substrates (Figure 5, Table 2). With UTP as the variable substrate, both reaction products, UDP and dCMP, were competitive inhibitors. These studies were performed with 2 μM dCyd since the nucleoside produces inhibition at concentrations > 3 μM (Figures 1B and 2). When dCyd was studied as the variable substrate, UDP was an uncompetitive inhibitor while dCMP produced noncompetitive inhibition at a saturating concentration of UTP. Similar patterns of inhibition were obtained with dCMP and UDP with respect

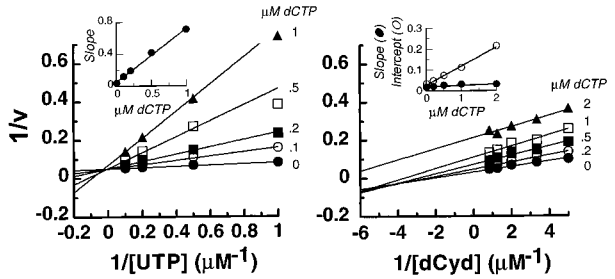


FIGURE 6: Inhibition of dCyd kinase by dCTP. (Left panel) UTP concentration was varied (1−10 μM) and dCyd was held constant at 2 μM. (Right panel) dCyd concentration was varied (0.3−1.2 μM) and UTP was held constant at 10 μM.

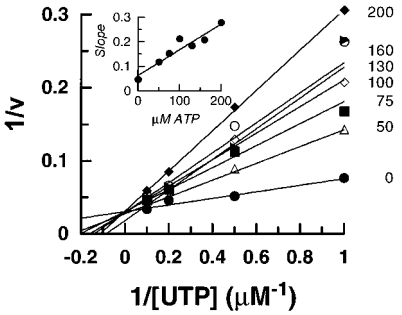
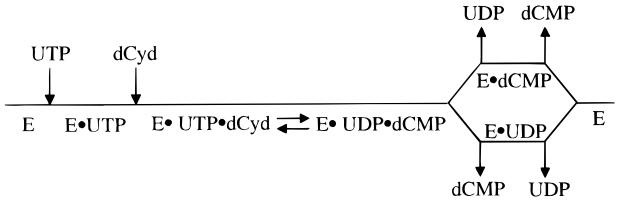


FIGURE 7: Inhibition of [^{32}P]UTP utilization by ATP. [^{3}H]dCyd was present at a constant concentration of 5 μM. Enzyme activity was measured as described in Materials and Methods based on the amount of [^{32}P]dCMP formed after separation by thin-layer chromatography.

to dCyd when the UTP concentration was increased to 100 or 2000 μM (data not shown).

The competitive pattern of inhibition between UDP and dCMP versus UTP indicate that these three compounds can bind to the same enzyme form, presumably free enzyme. This would suggest that the release of products is random, since both products compete with UTP for binding to free enzyme. Inhibition by dCMP or UDP versus dCyd clearly was not competitive, eliminating the possibility of random binding of substrates. The noncompetitive pattern with dCMP and the uncompetitive pattern of inhibition by UDP indicates that these compounds bind to enzyme forms distinct from that which binds dCyd. Taken together, the product inhibition data are consistent with an ordered pattern of substrate binding with UTP as the leading substrate, then binding of dCyd, followed by random release of the products dCMP and UDP. Consistent with this interpretation, the K_i values for inhibition of dCyd phosphorylation by dCMP or UDP increased at higher constant UTP concentrations (100 or 2000 μM; data not shown). We propose the following reaction sequence:

Scheme 1



The inhibition pattern with the dead-end inhibitor dCTP is also consistent with an ordered pattern of substrate addition. As illustrated in Figure 6, dCTP was a potent competitive inhibitor with respect to UTP with a K_i value of 0.07 μM, which is consistent with UTP as the first substrate to bind to the enzyme. With dCyd as the variable substrate, dCTP was a mixed inhibitor with K_{ii} and K_{is} values of 0.3 and 2.7 μM, respectively. This inhibition appears to be surmountable since the K_i value (with respect to dCyd) increased to 60 μM when the UTP concentration was raised to 2 mM (Shewach et al., 1992), again indicating that UTP is the leading substrate.

The substrate inhibition data are also consistent with this kinetic model. The noncompetitive pattern of inhibition by high concentrations of dCyd and the inability of high UTP concentrations to reverse the inhibition indicate that dCyd produces this inhibition by binding to a site other than the UTP binding site. Therefore, we propose that the substrate inhibition reflects the binding of dCyd prior to UTP, which is a less favorable order of substrate addition and results in a lower velocity. The finding that the inhibition by dCyd appeared to be only partial suggests that there is some randomness in the kinetic mechanism (Cleland, 1981). Consistent with this interpretation, we have reported previously that, with ATP as the phosphate donor, the dCyd kinase reaction follows a random bi-bi sequence (Datta et al., 1989b).

We also considered the possibility that the altered kinetics observed with UTP compared to ATP were due to the presence of an additional site on dCyd kinase to which UTP but not other nucleotides would bind. To test this possibility, an experiment was designed that employed 5 μM [^{3}H]dCyd, with [γ-^{32}P]UTP as the variable substrate and unlabeled ATP was added as an inhibitor of UTP utilization. As illustrated in Figure 7, these results demonstrated that ATP was a competitive inhibitor of UTP in this reaction, with an apparent K_i value of approximately 60 μM, similar to the observed K_m value of 54 μM for ATP (Table 2). The decrease in [^{32}P]dCMP formed from UTP was accompanied by an increase in the amount of [^{3}H]dCMP produced, indicating that ATP was able to displace UTP from the catalytic site and act as a phosphate donor for the reaction. The reciprocal experiment using [γ-^{32}P]ATP as the variable substrate with UTP as the inhibitor demonstrated that UTP was a potent competitive inhibitor of ATP utilization (K_i = 0.6 μM; data not shown). Thus, the data indicate that ATP and UTP compete for binding to the same site on the enzyme.

The parallel pattern of lines in the initial velocity kinetics was unusual for an enzyme with an ordered pattern of substrate addition. For either an ordered bi-bi or a rapid equilibrium random bi-bi system, the slope of the plot for $1/v$ vs 1/[A] is given by (Segel, 1975)

$$\text{slope} = (K_{ma}/V_{max})\,(1 + K_{ia}K_{mb}/K_{ma}[B])$$

The slope equation predicts that the double-reciprocal plots would produce a family of intersecting lines. However, if the dissociation constant for the first substrate (K_{ia}) is much less than its K_m value (K_{ma}), then the double-reciprocal plots would appear to be insensitive to changes in substrate concentration. In that case, the lines would appear to be parallel when in fact they intersect at a far distant point in the lower left quadrant (Segel, 1975). A similar rationale would produce seemingly parallel lines for the $1/v$ vs 1/[B] plot. With UTP as the proposed first substrate to bind, its dissociation constant, K_{ia}, was calculated on the basis of replots of the slope of ($1/v$ vs 1/[dCyd]) vs 1/[UTP] (data

from Figure 4), which yielded a value of approximately 0.09 μM, more than 13-fold lower than the corresponding K_m value. Calculation of dissociation constants for ATP, GTP, CTP, and dTTP indicated that each K_{ia} value was similar to its associated K_m value, with less than a 3.2-fold difference between corresponding values (Table 1). These data strongly suggest that the appearance of parallel lines in Figure 4 is due to a low dissociation constant for UTP relative to its K_m value.

DISCUSSION

Although ATP is commonly considered to be the universal phosphate donor for many phosphate-transferring enzymes, recent reports from this (Shewach et al., 1992) and other laboratories (White & Capizzi, 1991; Krawiec et al., 1996) demonstrate a compelling role for UTP as the true physiologic phosphate donor for dCyd kinase. The comparison presented here of true K_m and V_{max} values for cellular deoxy- and ribonucleoside 5′-triphosphates demonstrate that UTP is a 50-fold better substrate for dCyd kinase than ATP. Furthermore, of the six nucleotides tested, ATP was the poorest substrate. These data are consistent with a recent report indicating that the apparent K_m value for UTP was 15-fold lower than for ATP (Krawiec et al., 1996) and previous reports of the greater efficiency of nucleoside phosphorylation with UTP instead of ATP as the phosphate donor (White & Capizzi, 1991; Shewach et al., 1992). This first report of the true K_m value for UTP demonstrates clearly that, even with a 2-fold higher cellular concentration of ATP compared to UTP (Hauschka, 1973), dCyd kinase will utilize UTP 96% of the time as the phosphate donor. Thus, in previous studies with dCyd kinase employing a mixture of nucleotides including ATP and UTP as phosphate donors (White & Capizzi, 1991; Shewach et al., 1992; Krawiec et al., 1996), the kinetics of phosphorylation of the nucleoside substrate closely resembled that of UTP alone due to the 50-fold preference for UTP.

The choice of phosphate donors is important for dCyd kinase since the data presented here demonstrate that the kinetic mechanism is different when UTP is present compared to previous studies with ATP (Datta et al., 1989b). Previously we have reported that human dCyd kinase follows a rapid equilibrium random bi-bi reaction sequence when ATP was used as the phosphate donor (Datta et al., 1989b). In the presence of the preferred phosphate donor UTP, the product inhibition studies indicated that an ordered mechanism of substrate addition and a random release of products occurs with this enzyme. These results suggest that dCyd kinase can follow a random bi-bi reaction mechanism but, in the presence of UTP, the ordered addition of UTP followed by dCyd is strongly synergistic. Consistent with this interpretation, the noncompetitive substrate inhibition at high concentrations of dCyd, showing that the nucleoside inhibits by binding to a site other than that for UTP, and the observation that this inhibition was only partial all indicate that there is some randomness in the mechanism of substrate addition which results in a lower reaction velocity (Cleland, 1981). In contrast, in the presence of ATP there does not appear to be a preferred order of substrate addition and the random bi-bi reaction sequence predominates (Datta et al., 1989b).

A report on human dCyd kinase purified from human leukemic cells obtained from a patient concluded that the reaction followed an ordered bi-bi sequence with ATP as the phosphate donor (Kim & Ives, 1989). While the substrate addition sequence is similar to that reported here, product release distinguishes these two enzymes. The enzyme from the freshly isolated leukemic cells may represent a distinct protein since it appears to be a monomer (Kim et al., 1988) instead of the dimer used in our studies, which could account for the difference in mechanism. The monomeric enzyme also differs in that it did not exhibit substrate activation at high dCyd concentrations (Kim & Ives, 1989). Nonetheless, in view of the altered kinetics presented here using UTP as the phosphate donor, it may be interesting to determine whether the monomeric dCyd kinase exhibits a strong preference for the nucleotide substrate.

The family of parallel lines observed in the initial velocity plots only when UTP was used as the phosphate donor was unexpected for a mechanism that involves formation of a ternary enzyme−substrate complex but is likely explained by a low dissociation constant, K_{ia}, relative to the K_m value for UTP (Segel, 1975). The alteration in double-reciprocal plots with UTP but not other phosphate donors further confirms that UTP binds to the enzyme prior to dCyd. If dCyd were the first substrate to bind, then the dissociation constant would be independent of the binding of the nucleotide and no change in slopes of initial velocity data would be expected. A low dissociation constant relative to the K_m value has been suggested as the explanation for parallel lines with double-reciprocal plots of initial velocity kinetics for other enzymes that follow an ordered pattern of substrate addition (Baumann & Wright, 1968). Parallel initial velocity plots have been observed in random systems as well (Bar-Tana & Cleland, 1974; Gold & Segel, 1974; Bognar & Meighen, 1983), but the apparent ordered pattern of substrate addition shown here is inconsistent with such interpretations.

The data shown here demonstrated that dCTP was a potent inhibitor with respect to both dCyd and UTP when assays were performed with UTP concentrations near its K_m value. Previously we have reported a higher K_i value and an uncompetitive inhibition pattern for dCTP with respect to dCyd when UTP was the phosphate donor (Shewach et al., 1992). These results may be explained by a low dissociation constant for UTP. For a dead-end inhibitor that competes with the leading substrate for an enzyme with an ordered pattern of substrate addition, the slope of the double-reciprocal plot for inhibition against the second substrate is given by (Segel, 1975)

$$\text{slope} = (K_{mb}/V_{max})(1 + K_{ia}/[A] + K_{ia}[I]/K_i[A])$$

If the $K_{ia} \ll [A]$, then the slope will appear to be insensitive to changes in the concentration of inhibitor. Indeed, in Figure 6 the inhibition by dCTP with respect to dCyd showed a small change in the slope replot with increasing dCTP, indicated by a K_{is} that was more than 10-fold greater than the K_{ii} value. Thus, inhibition by dCTP with respect to dCyd may appear to be uncompetitive in the presence of saturating UTP when in fact it is noncompetitive. A similar rationale would also explain the apparent uncompetitive inhibition of dCyd by UDP with a constant UTP concentration. We have attempted to estimate the K_{ia} value for UTP directly, but the enzyme preparation was too dilute to measure this parameter accurately. Also, the purified enzyme contained micromolar

concentrations of UTP and dCyd that were used to elute dCyd kinase from a dCTP−Sepharose affinity column, further complicating measurement of the dissociation constant. In support of the concept of a low dissociation constant for UTP, we have noted that efficient removal of UTP from the enzyme preparation was difficult. Studies that measured the ability of the enzyme to phosphorylate [^{3}H]dCyd following double desalting indicated that the amount of [^{3}H]-dCMP formed was at least 8 times more than expected (average 97 ± 28; range 8−260) in 9 out of 12 experiments, based on the desalting efficiency of [^{3}H]UTP in the absence of enzyme. These data further indicate that UTP binds tightly to dCyd kinase, which appears to prevent its dissociation over a desalting column.

Results from this and other laboratories demonstrate that dCyd kinase is inhibited by its distal 5′-triphosphate end product dCTP (Datta et al., 1989b; Kim & Ives, 1989; White & Capizzi, 1991; Sarup et al., 1989; Shewach et al., 1992). However, the physiologic impact of this feedback inhibition appears to be small. Although these studies demonstrated potent inhibition by dCTP in the presence of UTP at concentrations near its K_m value, when UTP is present at physiologic concentrations (1−2 mM), dCTP is a relatively weak inhibitor of enzyme (K_i = 60 μM) (Shewach et al., 1992). Since cellular concentrations of dCTP do not normally exceed 20 μM (Hauschka, 1973), it is unlikely that dCTP can inhibit dCyd kinase significantly in the presence of 1−2 mM UTP concentration in intact cells. Other endogenous nucleotides have not been shown to have a regulatory effect on dCyd kinase. Interestingly, recent reports indicate that the nucleotide analog F-araATP, the 5′-triphosphate of the antileukemic drug 2-fluoroadenine arabinoside, can enhance phosphorylation of nucleoside analog substrates by dCyd kinase (Gandhi & Plunkett, 1988). Since this effect has been shown to be beneficial in chemotherapy with dCyd kinase substrates (Gandhi et al., 1994, 1995), the mechanism by which this occurs is currently under study.

An important kinetic difference with dCyd when UTP was used as the phosphate donor was the partial substrate inhibition. The data demonstrated that this inhibition was not caused by reaction products. That the inhibition was noncompetitive with respect to UTP and not reversible by higher UTP concentrations indicates that the inhibition is not due to dCyd binding at the UTP binding site. Taken together with the kinetic sequence results, these data suggest that high concentrations of nucleoside force dCyd kinase to bind dCyd first, resulting in a lower velocity. Alternatively, since the active enzyme is a dimer, it is possible that only half of the substrate binding sites are active catalytically, with the second site used in a regulatory function. In the presence of UTP, binding of dCyd at the second site, may produce substrate inhibition. With ATP, binding at the first site may decrease the affinity at the second site, resulting in negative cooperativity. Determining the stoichiometry of substrate binding as well as dissociation constants for all substrates will help to distinguish between these possibilities.

A previous report indicated that, with UTP as the phosphate donor, substrate inhibition occurs with the dCyd analog araC (White & Capizzi, 1991), and preliminary results suggest that a similar inhibitory effect is observed with dFdCyd (Shewach et al., 1994a). Reports from other investigators have demonstrated that phosphorylation of both araC and dFdCyd is saturable in human tumor cells *in vitro* or *in vivo* (Plunkett et al., 1987a; Muus et al., 1987; Grunewald et al., 1991; White & Capizzi, 1991; Abbruzzese et al., 1991; Shewach et al., 1994b). In those studies, concentrations of the analogs above 20 μM did not lead to increased accumulation of the cytotoxic triphosphates. The data presented here with dCyd suggest that binding of these analogs prior to UTP results in unfavorable kinetics with dCyd kinase in intact cells. Thus, these data provide additional rationale for the development of drug infusions designed to deliver nucleoside analogs at a dose that will allow efficient phosphorylation without causing inhibition of dCyd kinase (Plunkett et al., 1987b; Grunewald et al., 1990).

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BI970059R