Direct photoaffinity-labelling of human deoxycytidine kinase with the feedback inhibitor dCTP

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Deoxycytidine kinase (dCyd kinase, EC 2.7.1.74) is a key enzyme in the salvage pathway of deoxyribonucleosides, and the human enzyme is a dimer of two 30 kDa polypeptides with a broad substrate specificity, phosphorylating both purine and pyrimidine nucleosides and using various nucleoside triphosphates as phosphate donors. The enzyme is efficiently feedback-inhibited by dCTP, which presumably is the main regulator of its activity *in vivo*. Submicromolar concentrations of [³²P]dCTP could be used for direct photoaffinity labelling of pure dCyd kinase isolated from leukaemic spleen. A clearcut saturation of photoincorporation occurred with half-maximal incorporation at 0.07 μ M-dCTP. However, the total molar incorporation of dCTP was very low (approx. 0.1 %), in part due to a substantial u.v. inactivation of the enzyme. Proteinase digestion of labelled enzyme showed that dCTP was incorporated predominantly into a single peptide. Addition of equimolar concentrations of dCyd or dCMP as compared with dCTP inhibited photoincorporation approx. 50 %. The presence of other nucleoside substrates, as well as phosphate donors, also inhibited photolabelling of the enzyme. Thus photoincorporation of dCTP seems to occur at a site which can bind both the phosphate donors and acceptors of dCyd kinase, which strongly support the hypothesis that dCTP functions as a multi-substrate analogue, binding and bridging both substrate sites of the enzyme.

INTRODUCTION

Direct photoaffinity-labelling techniques have been used to cross-link nucleotides to several nucleotide-binding proteins, e.g. myosin [1], ribonucleotide reductase (EC 1.17.4.1) [2–6], DNA polymerase (EC 2.7.7.7) [7], deoxycytidylate deaminase (EC 3.5.4.12) [8] and terminal deoxynucleotidyltransferase (EC 2.7.7.31) [9], using both purine and pyrimidine nucleotides as photoaffinity ligands. These studies have demonstrated that dTTP and dCTP are particularly useful for specific and efficient photolabelling of enzymes involved in nucleotide metabolism. The structure of the amino acid–dTTP adduct formed during photolabelling of an allosteric site on ribonucleotide reductase has recently been fully characterized in this laboratory [10].

Deoxycytidine kinase (dCyd kinase; NTP:deoxycytidine-5'-phosphotransferase, EC 2.7.1.74) catalyses the phosphorylation of dCyd to dCMP in the presence of an NTP donor. The enzyme was studied after partial purification by many groups [11–20]. A purification procedure that yields homogeneous enzyme and uses leukaemic spleen as starting material has recently been developed in this laboratory. The active enzyme was a dimer of two 30 kDa subunits, and we found that it could efficiently phosphorylate both pyrimidine and purine nucleosides and nucleoside analogues, using ATP or dTTP as phosphate donors [21].

dCTP, the end product of the salvage pathway initiated by dCyd kinase, was a very efficient inhibitor of the pure enzyme, in agreement with the results of earlier studies [14,22], and dCTP probably serves as the physiological feedback regulator. The kinetic analysis of this inhibition showed competition between ATP and dCTP, whereas dCyd and dCTP were non-competitive [21].

Here we report on the specific photoaffinity-labelling of pure human dCyd kinase with dCTP. We found that both phosphate acceptors and phosphate donors could prevent labelling of the

30 kDa polypeptide in concentrations proportional to the K_m or K_i values for the respective substrates in earlier enzyme kinetic experiments [21].

MATERIALS AND METHODS

Radioactive nucleotides were obtained from Amersham Corp. Cytoplasmic dCyd kinase was purified from leukaemic spleen by a method described previously [21], and enzyme after dTTP–Sephacrose chromatography was used (more than 90 % pure as judged by SDS/PAGE). Nucleotides and nucleosides were from Sigma. Adenosine 5'-[β -imido]triphosphate (p[NH]ppA) was from Boehringer Mannheim, and erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA) was a gift from Burroughs–Wellcome (Research Triangle Park, NC, U.S.A.).

Photoaffinity labelling

The method used was essentially as described by Eriksson *et al.* [2]. The photochemical reaction was performed in drops (50 μ l) on Parafilm in a Petri dish placed on ice with dCyd kinase (1–2 pmol, i.e. 30–60 ng), 20 mM-Tris/HCl buffer, pH 7.6, 2 mM-MgCl₂, 5 mM-dithiothreitol (DTT) and 0.2 mg of BSA/ml. The [α -³²P]dCTP (specific radioactivity 0.1 μ Ci/pmol) had more than 98 % of the radioactivity as nucleoside triphosphate and less than 5 % was degraded after 30 min in the standard photoaffinity reaction, as judged by polyethyleneimine–cellulose t.l.c. with known standards. The u.v. light source (UVS-54 Mineral Light Lamp; UV Products, San Gabriel, CA, U.S.A.) was placed 15 cm from the reaction solutions, and the dose was 10 μ J (100 ergs)/s per mm² at 254 nm (measured with a u.v. meter). After irradiation the protein from 30 μ l of the reaction solution was precipitated with 5 % (w/v) trichloroacetic acid and analysed by PAGE.

Abbreviations used: dCyd kinase, deoxycytidine kinase (EC 2.7.1.74); p[NH]ppA, adenosine 5'-[β -imido]triphosphate; EHNA, erythro-9-(2-hydroxy-3-nonyl)adenine; DTT, dithiothreitol; IC₅₀, concentration causing 50 % inhibition.

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SDS/PAGE and autoradiography

Separation of the proteins (i.e. dCyd kinase and BSA) was performed on an SDS/10% (w/v) polyacrylamide gel, as described by O'Farrell [23], and the gel was stained with Coomassie Brilliant Blue. After drying, the gel was exposed at -70°C to Amersham MP film in a Fuji EC-A cassette. The developed film was subjected to scanning densitometry on a Joyce-Loebl microdensitometer, followed by integration of the area under the 30 kDa dCyd kinase peak for intensity estimation. The exposure time was chosen for each film so that a linear range of absorbances of the photolabelled 30 kDa band was obtained. Peptides after proteinase digestion were separated on an SDS/18%-polyacrylamide gel, which was silver-stained as described by Meril *et al.* [24].

Assay of dCyd kinase activity

The enzymic activity of dCyd kinase was determined by a radiometric assay as described by Ives & Wang [25] at 37°C in a total volume of 0.2 ml containing 40 mM-Tris/HCl, pH 7.6, 30 ng of pure dCyd kinase, 1 mM- Na_2ATP , 1 mM- MgCl_2 , 10 mM-DTT, BSA (0.5 mg/ml) and 10 μM - $[\text{}^3\text{H}]\text{dCyd}$. The reaction was started by addition of enzyme and was stopped by spotting aliquots (50 μl) on DEAE discs (Whatman DE81) at 0.5, 15 and 30 min. The initial velocities of the reactions were determined by using linear-regression analysis.

RESULTS

Specific photoincorporation of dCTP into the 30 kDa subunit of dCyd kinase

Increasing the dCTP concentration from 0.01 to 2.6 μM gave an increasing molar incorporation into the dCyd kinase polypeptide with a plateau above 1 μM (Fig. 1), measured by densitometric scanning of autoradiograms after SDS/PAGE of the reaction products as described in the Materials and methods section. The data in the Figure have been normalized by dividing each value by the incorporation at 2.6 μM , so that the y-axis is in

Fig. 1. Photoaffinity labelling of dCyd kinase 30 kDa subunit at different concentrations of $[\text{}^{32}\text{P}]\text{dCTP}$

Pure dCyd kinase (40 nm) and various concentrations of $[\text{}^{32}\text{P}]\text{dCTP}$ (0.01–2.56 μM) were irradiated 30 min with u.v. light as described in the Materials and methods section. The experimental values for the amount of incorporation are here plotted in terms of relative incorporation, where the amount at 2.56 μM -dCTP is set to 100%. The curve shows the best-fit hyperbola obtained from the equation $y = 100 \times x/(x + 0.07)$, where y is the relative incorporation at x μM of dCTP. The inset shows a semi-logarithmic plot of the same data.

terms of relative incorporation. The maximal molar incorporation is very low: 1 mmol of dCTP bound/mol of dCyd kinase. The reason for this is discussed below.

The dCTP concentration at 50% of maximal incorporation is approx. 0.07 μM , a value derived from a hyperbolic curve fitted to the experimental values described in the legend to Fig. 1. Using the assumption that photolabelling at these concentrations of dCTP represents specific binding to dCyd kinase, we can use this value to estimate the K_d (approx. 0.06 μM), as was done previously by Kowalczykowski [26] with the recA protein and azido-ATP photolabelling.

The specificity of dCTP photoincorporation was also demonstrated by a low incorporation into BSA at this low concentration of dCTP, even though the amount of BSA is 725 times higher than that of dCyd kinase. At concentrations of dCTP between 1 and 20 μM a linear increase of incorporation into BSA is seen (Fig. 2). This most likely represents non-specific binding. A similar linear increase in the photoincorporation of dCTP into dCyd kinase was also observed at these high concentrations. Heat-inactivated enzyme (100 $^{\circ}\text{C}$ for 7 min) or pre-u.v.-irradiated enzyme (60 min on ice) did not incorporate detectable amounts of dCTP under standard irradiation conditions (results not shown). In the following experiments we chose dCTP concentrations of 1 or 2 μM to eliminate non-specific binding (i.e. less than 10% of total incorporation), but still employed ligand concentrations 20 to 40 times the apparent dissociation constant.

Incorporation of dCTP with time appeared to be linear for only 10 min of irradiation and showed a plateau after approx. 60 min (Fig. 3). The reason for this is most likely inactivation of the enzyme. The half-life of the activity during photolabelling was 27 min, in both the presence or absence of 1 μM -dCTP. If the BSA concentration was lowered from 0.2 to 0.01 mg/ml, the half-life was approx. 4 min (results not shown). The presence of 20 μM -ATP did not affect the inactivation, but when both dCTP (1 μM) and ATP (20 μM) were present, enzyme activity was stabilized and the half-life was 37 min (Fig. 3).

To determine whether dCTP photolabelling occurred at many or few sites on dCyd kinase, we digested labelled enzyme with chymotrypsin or *Staphylococcus aureus* V8 proteinase and analysed the resulting peptides by PAGE (Fig. 4). A fraction of intact 30 kDa dCyd kinase polypeptide still remained after 17 h of digestion, but several smaller peptides were observed with both proteinases after silver staining of the gel. Autoradiography revealed that only one 17 kDa chymotryptic peptide contained cross-linked dCTP and one, or a few, V8-proteinase-cleaved peptides (~14 kDa in molecular mass) were photolabelled in addition to the undigested enzyme (Fig. 4).

Inhibition of dCTP photoincorporation by the presence of nucleosides

Adding dCyd was clearly inhibitory to dCTP incorporation (Fig. 2), and the concentration causing 50% inhibition (IC_{50}) was 0.5 μM (Table 1). The K_m values for dCyd, AraC, dAdo and dGuo as substrates were 1, 20, 150 and 120 μM respectively, using 1 mM-ATP as phosphate donor [21]. Addition of cytidine, which is a very poor substrate ([17]; B. Kierdaszuk & S. Eriksson, unpublished work), had a much less inhibiting effect and the IC_{50} value for this compound was 980 μM . dUrd, which is not a substrate for dCyd kinase, gave no inhibition, even at a concentration of 1 mM. Addition of dThd also had no effect at a concentration of 640 μM (Table 1).

A different behaviour was seen with the substrates dAdo, dGuo and AraC. At low concentrations (10–160 μM for dAdo and dGuo, 1–20 μM for AraC) dCTP incorporation was stimulated up to 50%, but at higher concentrations an inhibition was seen (Fig. 5).

Fig. 2. Direct photoaffinity labelling of dCyd kinase with [32 P]dCTP and [32 P]dTTP

dCyd kinase subunit (60 ng) was labelled with [32 P]dCTP or [32 P]dTTP (0.1 μ Ci/pmol) in a buffer solution (containing 0.2 mg of BSA/ml) kept on ice and irradiated for 30 min. The samples were then treated and analysed as described in the Materials and methods section. The autoradiograms were exposed for 12 h (a, lanes 1–3), 18 h (a, lanes 4–10) and 14 h (b). (a): lanes 1–3, variable concentrations of dCTP (5, 10 or 20 μ M); lanes 4–10, with 1 μ M-dCTP [lane 4 is with the addition of 1 mM-dAdo, lanes 5–9 are with the addition of dCyd (1, 2, 3, 5 and 10 μ M respectively) and lane 10 is without addition of competing nucleoside]. (b) 2 μ M-dTTP was used as photoligand; in lanes 1 and 2, 5 mM-ATP was added; in lanes 3 and 4, 100 μ M-ATP was present; in lanes 5 and 6, both 100 μ M-ATP and 10 μ M-dCTP were present during the reaction; lane 7 shows photolabelling with dTTP and without the addition of competing ligand. In (c) a Coomassie Brilliant Blue-stained gel (corresponding to a, lanes 5–10) is shown; lanes 7 and 8 show 0.3 and 0.5 μ g of unirradiated dCyd kinase, with the addition of 6 μ g of BSA in lane 8. The arrows indicate the position of the dCyd kinase polypeptide. As molecular-mass markers (M), phosphorylase *b* (94 kDa), BSA (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa) and α -lactalbumin (14.4 kDa) were used.

Fig. 3. Time-dependence of photoaffinity-labelling and u.v. inactivation of dCyd kinase

Enzyme and 2 μ M-[32 P]dCTP were irradiated with u.v. light for various lengths of time. The autoradiogram of the gel was subjected to scanning densitometry, and incorporation (in arbitrary units) was determined (■): dCyd kinase was irradiated in the absence (▲) or presence of 1 μ M-dCTP (◇), 20 μ M-ATP (□) or 1 mM-dCTP plus 20 μ M-dCTP plus 20 μ M-ATP (△), and the relative enzyme activity was determined (activity in the different mixtures without any irradiation was set to 100%).

Inhibition of dCTP photoincorporation in the presence of nucleotides

Addition of the product, dCMP, showed efficient inhibition of dCTP incorporation, with an IC_{50} value of 1 μ M, which was the same as the dCTP concentration in the experiments. dCDP was less effective, with an IC_{50} value of 75 μ M. The nucleotide analogue ddCTP, which is the end product of dideoxycytidine phosphorylation initiated by dCyd kinase, had an IC_{50} value of 15 μ M. CTP is, in contrast with dCTP, a phosphate donor for the enzyme, and its presence also inhibited dCTP incorporation (IC_{50} = 70 μ M). dTTP, which is an effective phosphate donor (K_m = 2 μ M [21]), was an efficient inhibitor, with an IC_{50} value of 3 μ M (Table 1).

dATP, dGTP and GTP can all serve as phosphate donors for the enzyme, and they were all equally effective in inhibiting dCTP incorporation, with IC_{50} values of 35 μ M (Table 1). The effect observed with ATP was similar to that observed with dAdo, dGuo and AraC. At low concentrations (1–20 μ M) a stimulation was observed, but, at higher concentrations of ATP, this stimulation turned to an inhibition, with a 50% effect at 160 μ M. The ATP analogue p[NH]ppA showed a similar effect at low concentrations, but was a less effective inhibitor, with an IC_{50} value of 670 μ M. This analogue does not serve as a phosphate donor, owing to the stable N–P bond between the β - and γ -phosphate groups.

Fig. 4. Peptide mapping of [^{32}P]dCTP-labelled dCyd kinase

Enzyme (1 μg) and BSA (10 μg) were irradiated together with 1 μM dCTP for 20 min as described in the Materials and methods section, precipitated with acid and, after washing the precipitate with acetone, dissolved by boiling for 2 min in 0.125 M-Tris/HCl (pH 6.8)/0.5% SDS/10% (v/v) glycerol. One fraction (20%) was undigested (lanes 1 and 2), and two further (equal) fractions (40%) were incubated with chymotrypsin (20 $\mu\text{g}/\text{ml}$) (lanes 3 and 4) or *S. aureus* V8 proteinase (13 $\mu\text{g}/\text{ml}$) (lanes 5 and 6) for 16 h at 37 $^{\circ}\text{C}$. Lanes 1, 3, 5 and 7 shows a photograph of the silver-stained gel, and lane 7 contains protein markers as in Fig. 2. Lanes 2, 4 and 6 show an autoradiogram of the gel exposed for 10 h. An arrow indicates the position of undigested dCyd kinase.

Table 1. Effects of nucleosides and nucleotides on the photoincorporation of 1 μM -[^{32}P]dCTP into dCyd kinase

The concentrations giving 50% inhibition of photoincorporation (IC_{50}) were calculated from the results of experiments such as that described in Fig. 5. The values are means of measurements from at least two experiments, and the individual measurements differed less than 25% from the mean.

Addition	IC_{50} (μM)
Nucleosides	
dCyd	0.5
AraC	160
dAdo	900
dGuo	1200
Cyd	980
Ado	≥ 640
dThd	≥ 640
dUrd	≥ 1000
Nucleotides	
ATP	160
dATP	35
p[NH]ppA	670
ADP	230
GTP	35
dGTP	35
dTTP	3
CTP	70
ddCTP	15
dCDP	75
dCMP	1

Photoaffinity labelling with [^{32}P]dTTP

With dTTP as a photoaffinity ligand we found a much higher non-specific incorporation into BSA, even at low concentrations of dTTP ($\sim 1 \mu\text{M}$) (Fig. 2). However, specific incorporation of dTTP into dCyd kinase was also observed. Addition of dCTP to a reaction mixture containing labelled dTTP (2 μM) gave in-

Fig. 5. Effect of different nucleoside and nucleotide additions on dCTP incorporation

Enzyme and 1 μM -[^{32}P]dCTP alone or after addition of Ado ($\circ$) dAdo ($\square$) or dATP ($\blacktriangle$) were u.v.-irradiated for 30 min. Incorporation of dCTP into the 30 kDa polypeptide with no addition is set to 100%.

hibition of dTTP incorporation with an approximate IC_{50} value of 1 μM . ATP also had an inhibitory effect: 100 μM -ATP (i.e. 50 times higher concentration than the dTTP concentration) gave 50% inhibition.

DISCUSSION

Here we describe the first binding study with dCyd kinase and dCTP, the presumed physiological feedback inhibitor, using a direct photoaffinity-labelling technique. Evidence for the specificity of the photolabelling is as follows: (i) saturation of the photo-cross-linking at submicromolar concentration, close to the K_i value determined kinetically (see below); (ii) only active dCyd kinase incorporated dCTP; (iii) additions of nucleotides and nucleosides known to interact with dCyd kinase inhibited photolabelling, whereas nucleosides known to have no effect on the enzyme had no effect on the photolabelling; (iv) proteinase digestion of labelled enzyme showed that only one (or a few) peptide(s) appeared to be cross-linked to dCTP.

The low molar incorporation of dCTP into dCyd kinase is most likely due to the fact that, in direct photolabelling reactions, only a small fraction of the interactions between the natural ligand and the enzyme leads to cross-linking, since only a small proportion of the nucleotide is photo-induced to a reactive state. This is a general problem with direct photoaffinity-labelling methods, as discussed by Shetlar [27]. Furthermore, there is a rapid u.v. inactivation of the enzyme during the photolabelling reaction, especially in the absence of added carrier protein, which prevents the use of longer labelling times. With dCyd kinase in the presence of BSA, dCTP seems to be a more specific photoligand than dTTP, although the photoincorporation of dTTP into dCyd kinase also appeared to occur at the same site, as judged by its competition with dCTP.

The cross-linking of dCTP to dCyd kinase followed a simple hyperbolic binding curve, with good fitting also to an exponential form of this theoretical curve. The calculated apparent K_d value obtained using the half-maximal concentration (70 nM) from this curve was 60 nM, which agrees well with the K_i value (30 nM) obtained in competition experiments with varying ATP, a fixed dCTP concentration, and using dCyd as substrate [21].

We observed a stimulation of photoincorporation at around micromolar concentrations of several nucleosides and nucleotides (Fig. 5), and we attribute this effect to the partial stabilization of the enzyme, as was observed when both ATP and dCTP were

present (Fig. 3). These results indicate that both ligands, although interacting with the same functional site, can bind simultaneously to the enzyme, possibly at different domains of the active site.

dCyd (and the product dCMP) prevents the cross-linking in concentrations equimolar with that of dCTP. Other nucleosides (i.e. AraC, dGuo and dAdo) inhibit the photoincorporation, but at higher concentrations that correspond approximately to their respective K_m values [21]. The discrepancy between the apparent affinities of dCyd and the other nucleoside substrates for the enzyme was previously suggested to be dependent on the presence of different conformational forms of dCyd kinase: one form specific for dCyd and one form for purine deoxyribonucleosides [21]. We are forced to conclude from the photolabelling results that dCTP induces and binds to the dCyd-specific form of the enzyme.

Nucleoside triphosphates which serve as phosphate donors for the enzyme also prevented photolabelling. The concentration of dTTP necessary to inhibit dCTP cross-linking is approx. 20-fold lower than its K_m value as phosphate donor. The effect of ATP shows less discrepancy between the approximate K_d measured by inhibition of dCTP and its apparent K_m value ($\sim 10 \mu\text{M}$; [21]). However, the result with ATP is complicated by the observed stabilization during u.v. irradiation. Other methods to determine the interaction between ATP and dCyd kinase is needed to clarify this point.

Ikeda *et al.* [29] have proposed a general mechanism for end-product inhibition of deoxynucleoside kinases based on kinetic studies with the *Lactobacillus acidophilus* enzyme using multi-substrate analogues (i.e. deoxynucleoside 5'-adenosine 5'-p¹,p⁴-tetraphosphates). They suggest that dNTPs serve as multi-substrate inhibitors where the deoxynucleoside moiety of the nucleotide fits the deoxynucleoside-binding phosphate-acceptor site and the charged triphosphate group interacts with an adjacent phosphate-donor-binding site on the enzyme. A prediction of this model is that the combination of two sets of binding determinants results in much tighter binding than that exhibited by either substrate alone. Recent kinetic studies with purified human dCyd kinase by Kim & Ives [30] have demonstrated that the model appears to be applicable also to the human enzyme.

The fact that all known phosphate acceptors and donors of dCyd kinase inhibited the photoincorporation of the end product dCTP supports this elegant and simple mechanism. Furthermore, the competition experiments show that nucleotides that can bind via interaction with both sites on the enzyme generally have a lower IC_{50} value than nucleotides bound only to the phosphate donor subsite. However, there are also other co-operative allosteric effects involved in the substrate regulation of dCyd kinase,

and further studies need to be aimed at clarifying the nature of these processes.

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