Selective Inactivation of the Deoxyadenosine Phosphorylating Activity of Pure Human Deoxycytidine Kinase: Stabilization of Different Forms of the Enzyme by Substrates and Biological Detergents†

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ABSTRACT: Deoxycytidine kinase, purified from human leukemic spleen to apparent homogeneity, is a multisubstrate enzyme that also phosphorylates purine deoxyribonucleosides [Bohman & Eriksson (1988) *Biochemistry 27*, 4258–4265]. In the present investigation we show that the stability and temperature dependence of dCyd kinase activity differed appreciably from the dAdo kinase activity of the same pure enzyme. Selective inactivation of dAdo activity was observed upon an incubation of the enzyme at both 4 and 37 °C. The half-life of dAdo activity at 4 °C increased from 36 to 84 h, when the protein concentration was increased by addition of bovine serum albumin. However, the half-life of dCyd activity increased from 72 h to more than 7 days under the same conditions. dCyd activity was stable for at least 6 h at 37 °C while the half-life of dAdo activity was 2 h. The presence of substrates like ATP, dTTP, or dAdo stabilized dAdo activity at both temperatures, and full maintenance of both activities at 37 °C was obtained by the addition of the zwitterionic detergent CHAPS. Furthermore, thermal inactivation of the dAdo activity occurred at a lower temperature (48 °C) as compared to the dCyd activity (54 °C). The presence of protease inhibitors had no effect on enzyme inactivation, nor was there a difference in the subunit structure of the selectively inactivated enzyme as compared to the fully active form, as revealed by size-exclusion chromatography. Unexpectedly, it was found that biological detergents like CHAPS and sodium cholate were competitive inhibitors of dAdo phosphorylation and weak stimulators of dCyd phosphorylation without changing the overall subunit structure of the enzyme. Our data can explain the varying substrate specificity reported for dCyd kinase in earlier studies and strongly suggest that the enzyme exists in different conformational state(s) with different substrate kinetic properties.

Deoxycytidine kinase (dCyd kinase)[1] (NTP:deoxycytidine-5′-phosphotransferase, EC 2:7.1.74) catalyzes the phosphorylation of 2′-deoxycytidine (dCyd) to 2′-deoxycytidine 5′-monophosphate (dCMP) in the presence of a nucleoside 5′-triphosphate phosphate donor. The cytosolic enzyme has been isolated and partially purified from many sources (Kessel, 1968; Momparlier & Fisher, 1968; Durham & Ives, 1970a,b; Kazai & Sugino, 1971; Coleman et al., 1975; Krenitsky et al., 1976; Cheng et al., 1977; Meyers & Kreis, 1976; Hurley et al., 1983; Sarup & Fridland, 1987; Datta et al., 1989). Recently, dCyd kinase has been purified to apparent homogeneity from human leukemic spleen (Bohman & Eriksson, 1988) and leukemic human T-lymphoblasts (Kim et al., 1988). Most investigators have found that the same enzyme also phosphorylated purine deoxyribonucleosides, including several nucleoside analogues.

The kinetic behavior of the enzyme has been reported to be very complex, exhibiting cooperative effects with ATP as phosphate donor and dCyd as phosphate acceptor (Ives & Durham, 1970a,b; Sarup & Fridland, 1987). A different kinetic pattern was observed with other nucleosides, e.g. arabinosylcytosine (*ara*-C), 2′-deoxyguanosine (dGuo), and 2′-deoxyadenosine (dAdo) (Durham & Ives, 1969; Ives & Durham, 1970a,b; Sarup & Fridland, 1987). Recently, we have established that pure dCyd kinase, purified from human leukemic spleen (Bohman & Eriksson, 1988), exhibits substrate-induced negative cooperativity with dCyd, dAdo, and ATP as well as with *ara*-C (C. Bohman, B. Kierdaszuk, and S. Eriksson, unpublished results). Furthermore, there are considerable differences reported in the literature regarding the substrate specificity of dCyd kinase from various sources. For instance, cytoplasmic dCyd kinase from human leukemic cells was shown to be specific for dCyd and *ara*-C (Coleman, 1975; Cheng et al., 1977). Two human placental dCyd kinases capable of phosphorylating dAdo or dGuo, but not both, have been described by Hurley et al. (1983). An apparently homogeneous murine dCyd kinase, characterized in detail, was unable to phosphorylate purine 2′-deoxyribonucleosides (Meyers & Kreis, 1976, 1978).

The active form of the cytoplasmic dCyd kinase from human leukemic spleen appeared to be a dimer of two 30-kDa subunits, and it phosphorylated dCyd, *ara*-C, dAdo, and dGuo, with ATP or dTTP as phosphate donors (Bohman & Eriksson, 1988). The enzyme can phosphorylate 2′,3′-dideoxynucleoside analogues, with potential use in AIDS therapy (Kierdaszuk et al., 1989). We have shown that cytosine-containing analogues, i.e., dideoxycytidine, 2′,3′-didehydrodideoxycytidine, and the acyclic analogue N^1-(4′-hydroxy-1′,2′-butadienyl)cy-

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[1] Abbreviations: dCyd kinase, deoxycytidine kinase; BSA, bovine serum albumin; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; DTT, dithiothreitol; Tris-HCl, tris(hydroxymethyl)-aminomethane hydrochloride; dCyd, dAdo, dGuo, and dThd, 2′-deoxyribonucleosides of cytosine, adenine, guanine, and thymine, respectively; NTP, nucleoside triphosphate.

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tosine (cytalene), were efficiently phosphorylated, while unexpectedly, no activity was found with purine dideoxynucleosides. During further attempts to study the activity of this enzyme toward a large number of nucleoside substrates, it was observed that the ratio of dAdo to dCyd activity decreases upon storage of the enzyme on ice. In this paper, we characterize this phenomenon, and our results may explain the varying substrate specificity reported earlier for different preparations of dCyd kinase. In addition, we provide evidence for the existence of different active forms of this complex enzyme.

Materials and Methods

Materials. [5-^{3}H]-2′-Deoxycytidine (19.3 Ci/mmol) and [G-^{3}H]-2′-deoxyadenosine (29 Ci/mmol) were purchased from the Radiochemical Centre, Amersham. Unlabeled nucleosides, ATP (disodium salt), and bovine serum albumin (fraction V) were obtained from Sigma Chemical Co., and dTTP (trisodium salt) was from Pharmacia LKB Biotechnology, Sweden. The anionic detergent cholic acid (sodium salt) was from Sigma, and two zwitterionic detergents, *N*-tetradecyl-*N*,*N*-dimethyl-3-ammonio-1-propanesulfonate (zwittergent 3-14) and 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), were purchased from Calbiochem and Boehringer Mannheim, respectively. The detergents were made up as 0.1 M stock solutions in water.

The TSK-G3000SW column was purchased from Pharmacia LKB Biotechnology, Sweden. The calibration proteins, size I, for column calibration were from Boehringer Mannheim. All other chemicals, reagents, and materials were of the highest quality commerically available.

Deoxycytidine kinase was purified from human leukemic spleen as described by Bohman and Eriksson (1988).

Kinetic Parameters. dCyd kinase activity was routinely followed by a radiochemical assay procedure described by Ives and Wang (1978). Unless otherwise indicated, assays were performed at 37 °C in a total volume of 200 μL containing 40 mM Tris-HCl, pH 7.6, 1 mM ATP, 1 mM $MgCl_2$, 0.1 M KCl, 50–80 ng of pure dCyd kinase, 0.5 mg/mL BSA, and a radiolabeled nucleoside at the concentrations indicated. The reaction was initiated by addition of enzyme and terminated by spotting aliquots (40 μL) onto DEAE filter disks (Whatman DE-81, 25-mm diameter) at 0.5, 10, 20, and 30 min, respectively. The disks were immediately washed three times for 30 min with deionized water during the assay of dCyd activity or with 1.5 mM ammonium formate during the assay of dAdo activity. The remaining products were eluted with a 0.5-mL mixture of 0.2 M KCl/0.1 M HCl (1:1 v/v) and quantitated by liquid scintillation. Initial reaction velocities and kinetic parameters were determined by using linear regression analysis.

Hill plots were drawn according to the Hill equation (Dixon & Webb, 1979). An apparent maximal velocity (V_m) of the enzyme reaction was obtained from an Eadie–Hofstee plot of the kinetic data in the range of high substrate concentration (S). The Hill coefficients were determined by using linear regression analysis of the experimental points in the substrate concentration range where reaction velocity was higher than 10% and lower than 90% of the saturation velocity.

Gel Filtration Chromatography. Analytical gel filtration chromatography was performed on a TSK-G3000SW (600 × 7.5 mm) column using an LKB 2150 HPLC pump, with a Rheodyne 7125 injector and two Shimadzu SPD-2A detectors operating at 280 and 260 nm. The column was equilibrated and eluted at a flow rate of 0.1 mL/min by using 50 mM Tris-HCl (pH 7.6) buffer containing 5 mM $MgCl_2$, 1 mM DTT, and 0.1 M KCl. The fraction volume was 0.2 mL. Catalase (M_r 240 000), BSA (M_r 67 000), ovalbumin (M_r 43 000), and chymotrypsinogen A (M_r 25 000) were used as molecular weight markers for column calibration. The elution volume of the standard protein was determined by following the difference in absorption at 280 and 260 nm. Determination of dCyd and dAdo phosphorylation and SDS–polyacrylamide gel electrophoresis, performed as described by O'Farrel (1975), were used to detect the elution volume of dCyd kinase. The polypeptide bands were visualized by using a positive-image silver stain (Merril et al., 1984).

Results

Selective Inactivation of dCyd Kinase. Kinetic results with dCyd kinase show a biphasic saturation curve with dCyd and dAdo as substrate (Ives & Durham, 1970a,b; Sarup & Fridland, 1987; Bohman et al., unpublished results). A model with two K_m and V_{max} values has been used to describe this type of enzyme kinetics. In this investigation we chose to use two concentrations of dCyd, 2 and 100 μM, to detect possible differential effects on these parameters at the high and low concentration of dCyd. The first concentration is approximately equal to the low K_m value, and the second one is approximately 10 times the high K_m value. To measure dAdo activity, we use only a 100 μM concentration, which is in the range of the low K_m value of this substrate (Bohman et al., unpublished results).

Figure 1 shows that there was a substantial difference in the stability of dAdo phosphorylating activity of the enzyme (with a half-life of 1.5 days) as compared to that with the two dCyd concentrations (with a half-life of 3.2 and 2.5 days at 2 and 100 μM, respectively) upon incubation at 4 °C at 0.02 mg/mL BSA (Figure 1A). Addition of BSA up to 0.5 mg/mL (Figure 2B) stabilized the activities so that no dCyd activity decrease was seen in 6 days while dAdo phosphorylation activity had a half-life of 3.3 days (Figure 2B). A series of experiments was set up to test other stabilizing factors. First of all, there was an effect of the enzyme concentration in the incubation mixture. When enzyme concentration decreased from 4 to 1 μg/mL in 40 mM Tris-HCl (pH 7.6) containing 0.52 mg/mL BSA, enzyme activity toward dCyd and dAdo (both at 100 μM) had a lower stability; i.e., after 48-h incubation at 4 °C they were 72 and 38% of the initial values, instead of 100 and 67%, respectively, at higher concentration of the enzyme. The presence of the reducing agent DTT (10 mM) and/or 20% glycerol and/or 0.1 M KCl had no protective effect. Addition of substrates, i.e., ATP (0.5 mM), dTTP (0.1 mM), or dAdo (0.45 mM), conferred a stabilizing effect on the enzyme when added separately. The effect of dCyd was not tested. We found with 1 μg/mL enzyme at 4 °C in 40 mM Tris-HCl buffer (pH 7.6) containing 0.52 mg/mL BSA, 4% glycerol, 1 mM $MgCl_2$, and 0.3 mM DTT that, after 48-h incubation enzyme, activity toward dCyd and dAdo remained at an average level of 88 ± 5% and 72 ± 10%, respectively, while it was 72 and 38% in the control sample containing no substrates.

A similar pattern was seen when the enzyme (4 μg/mL) was incubated at 37 °C in the presence of 0.13 or 0.63 mg/mL BSA, but in this case the time scale of the process was in the range of hours. The half-life of dCyd activity was longer than 6 h (Figure 2A) and for dAdo activity about 2 h (Figure 2B). There was a significant stabilizing effect with 0.25 mM CHAPS and dAdo as substrate, giving a half-life longer than 6 h (Figure 2). Since CHAPS also competitively inhibited dAdo phosphorylation (see below), enzyme samples had to be diluted to reduce the detergent concentration before measuring the dAdo activity. Addition of substrates, i.e., ATP, dTTP,

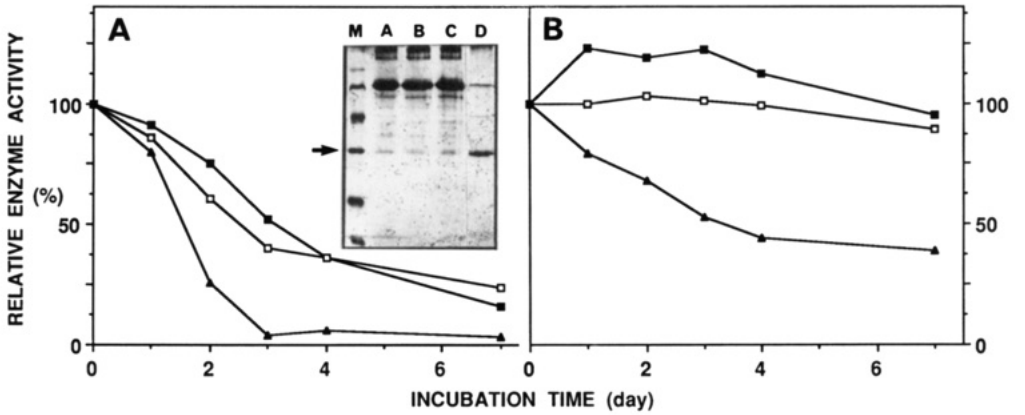

FIGURE 1: Time course of the dCyd kinase activity toward dCyd at 2 μM (□) and 100 μM (■), and 100 μM dAdo (▲), with BSA at 0.02 mg/mL (A) or 0.52 mg/mL (B). Enzyme (4 μg/mL) was incubated at 4 °C in 40 mM Tris-HCl buffer (pH 7.6) containing 4% glycerol, 1 mM $MgCl_2$, and 0.3 mM DTT. Control values were 20 and 50 nmol/(min·mg) for the two dCyd concentrations and 320 with dAdo as substrate. The insert shows 12% SDS gel electrophoresis of the same enzyme samples containing 0.1 μg of dCyd kinase and 0.5 μg of BSA per lane: prior to incubation (lane A); after 3 days of incubation at 4 °C in the absence (lane B) and in the presence (lane C) of eight protease inhibitors (see text). The enzyme (0.6 μg) without BSA added was also run for comparison (lane D). The polypeptide bands were developed by using silver staining. The arrow indicates the position of the 30-kDa polypeptide of the dCyd kinase. Phosphorylase *b* (64 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa), and α-lactalbumin (14.4 kDa) were used as a molecular mass markers (lane M).

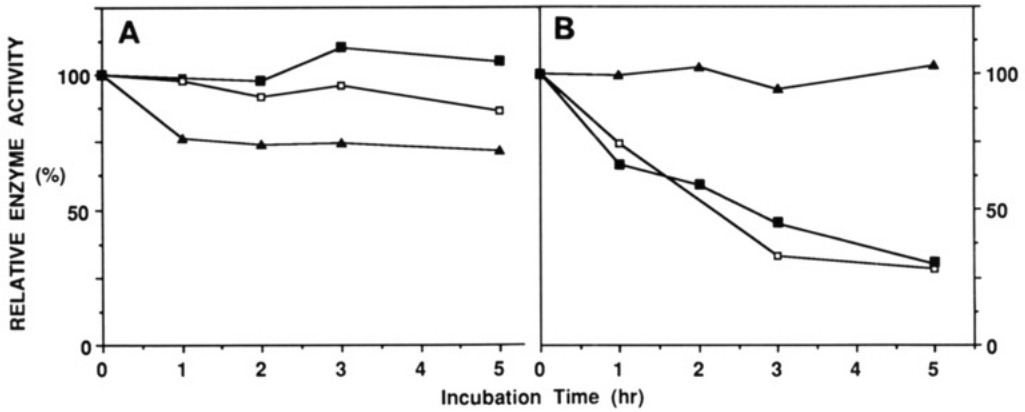

FIGURE 2: Time course of the dCyd kinase activity toward 5 μM dCyd (A) and 200 μM dAdo (B) with 0.125 mg/mL BSA (■); 0.625 mg/mL BSA (□); or 0.125 mg/mL BSA and 0.25 mM CHAPS (▲). Enzyme (5 μg/mL) was incubated at 37 °C in 40 mM Tris-HCl buffer (pH 7.6) containing 1.25 mM $MgCl_2$, 5% glycerol, and 0.25 mM DTT. Control values for the three different conditions were 27, 28, and 32 nmol/(min·mg) with dCyd and 495, 487, and 421 nmol/(min·mg) with dAdo as substrate.

or dAdo, also significantly stabilized the activity vs dAdo (Table I).

In an attempt to characterize the nature of the selective inactivation process, we investigated if the presence or protease inhibitors would stabilize the dAdo activity. Incubation of dCyd kinase (4 μg/mL) for 72 h at 4 °C in 40 mM Tris-HCl (pH 7.6) buffer containing 1 mM phenylmethanesulfonyl fluoride, 5 mM benzamidine, 5 mM 1,10-phenanthroline, 0.1 mM diphenylcarbamyl chloride, 1 mg/mL aprotinin, 10 μg/mL leupeptin, 0.1 mg/mL agmatin, and 1 μM bestatin led to a 62% decrease of dAdo activity, as compared to 78% in the control sample which did not contain any protease inhibitors. There appeared to be no significant change in the polypeptide pattern on SDS gel analysis, i.e., in the intensity of the 30-kDa human dCyd kinase protein (Bohman & Eriksson, 1988) after 72-h incubation at 4 °C either in the presence or in the absence of protease inhibitors (Figure 1A, insert). A similar experiment performed at 37 °C showed no change in the SDS gel electrophoresis pattern during a 6-h incubation (data not shown). We thus found no evidence for proteolysis of enzyme during the incubation which could be related to the selective inactivation of dAdo activity.

The kinetic properties of the enzyme were studied after 3 days of incubation at 4 °C and compared to unincubated enzyme from the same preparation. We found that the negative cooperativity of dCyd phosphorylation increased, as shown in a Hill plot (Figure 3), so that the Hill coefficients were 0.35 ± 0.08 and 0.6 ± 0.1 for incubated and unincubated enzyme samples, respectively. This was accompanied by a

Table I: Stabilizing Effect of Substrates on dCyd Kinase Activity toward 5 μM dCyd and 200 μM dAdo

incubation conditions[a]	act. remaining after 2.5-h incubation at 37 °C[b] (% of initial act.)	
	dCyd	dAdo
control	103	38
+1.0 mM ATP	112	65
+0.5 mM dTTP	104	91
+1.0 mM dAdo	107	100
+0.25 mM CHAPS	105	111

[a] dCyd kinase (5 μg/mL) was incubated 2.5 h at 37 °C in 40 mM Tris-HCl and 50 mM phosphate buffer (pH 7.6) containing 1.25 mM $MgCl_2$, 5% glycerol, 0.25 mM DTT, and 0.625 mg/mL BSA without (control sample) and in the presence of substrates and CHAPS detergent, as indicated. [b] Assay conditions were as described under Materials and Methods, except that incubated enzyme samples were diluted 5-fold for assay and 5 μM dCyd or 200 μM dAdo was used as substrate. The values are the mean of three separate determinations, varying by less than 10% of the mean value. Control valuls were as follows: for dCyd, 25 (without and with ATP), 34 (with dTTP), 28 (with dAdo), and 37 nmol/(min·mg) (with CHAPS), and for dAdo with the same additions, 498, 576, 502, and 447 nmol/(min·mg), respectively.

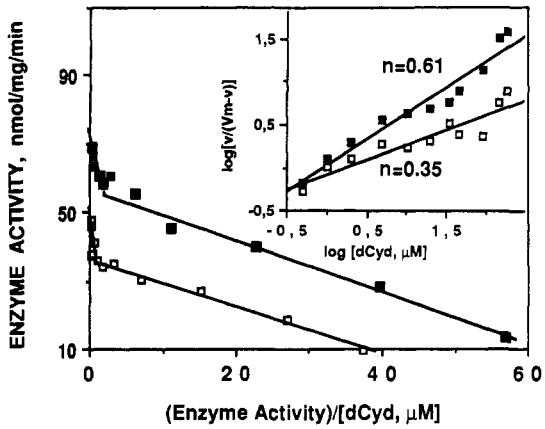

FIGURE 3: Eadie–Hofstee plot of the dependence of dCyd kinase activity on dCyd concentration for unincubated enzyme (■) and for 72 h incubated enzyme (□), i.e., with dAdo activity selectively inactivated to 50% of that for unincubated enzyme, with 1 mM ATP as phosphate donor. Insert shows a Hill plot of the same data together with Hill constants for the unincubated ($n = 0.6 \pm 0.1$) and incubated enzyme ($n = 0.35 \pm 0.08$), respectively. For selective inactivation, enzyme (4 μg/mL) was preincubated 72 h at 4 °C in 40 mM Tris-HCl buffer (pH 7.6) containing 0.52 mg/mL BSA, 4% glycerol, 1 mM $MgCl_2$, and 0.3 mM DTT.

change in the V_{max} value from 70 to 54 nmol/(min·mg). When dAdo was used as a substrate (at 5–500 μM), there was about 3-fold decrease in the V_{max}, from 702 to 254 nmol/(min·mg), only a 20% decrease in the K_m value, from 52 to 42 μM, and no change in the Hill coefficient, which at these dAdo concentrations is approximately 1.

In order to investigate the effect of selective inactivation on the overall subunit structure of dCyd kinase, we analyzed inactivated enzyme with the aid of HPLC size-exclusion chromatography. In both cases dCyd kinase was eluted as a single symmetrical peak at the position of 60 kDa, as determined by activity assays and SDS gel electrophoresis. With both incubated and unincubated enzyme dAdo activity followed exactly the dCyd activity, measured with both 250 μM nucleoside and 1 mM ATP as phosphate donor. The SDS gel electrophoresis showed that the intensity of the 30-kDa polypeptide followed both enzyme activities and no 30-kDa protein was detected at the position of either a tetramer or monomer form of the enzyme (data not shown).

Temperature Dependence of Enzyme Activity. The activity of pure enzyme at different temperatures with two concentrations (5 and 250 μM) of dCyd and 250 μM dAdo is shown in Figure 4A. The reasons for using different concentrations of substrates were mentioned in the preceding section. The enzyme sample, containing 40 mM Tris-HCl (pH adjusted to 7.6 at each temperature), 0.5 mg/mL BSA, and 4 μg/mL dCyd kinase, was preincubated for 5 min at each temperature and its activity measured at that temperature. The rapid drop in the reaction rate, due to thermal inactivation of the enzyme, occurred at different temperatures for dCyd and dAdo phosphorylation activities (Figure 4A), i.e., at 54 and 48 °C, respectively. The temperature optimum for dCyd activity was 50 °C while it was 44 °C for dAdo activity. dAdo activity exhibited an 80% decrease at 50 °C (Figure 4A). When the data were plotted in the form of an Arrhenius plot (Figure 4B), the apparent activation energy values were determined by linear regression analysis of the linear part of the curves, i.e., between 4 and 44 °C for dCyd activity and between 4 and 31 °C for dAdo activity. They were 17.1 ± 0.8 and 15.5 ± 0.6 kcal/mol for dAdo and dCyd phosphorylation, respectively.

Effects of Biological Detergents. To increase the yield and stability of dCyd kinase during purification, we investigated

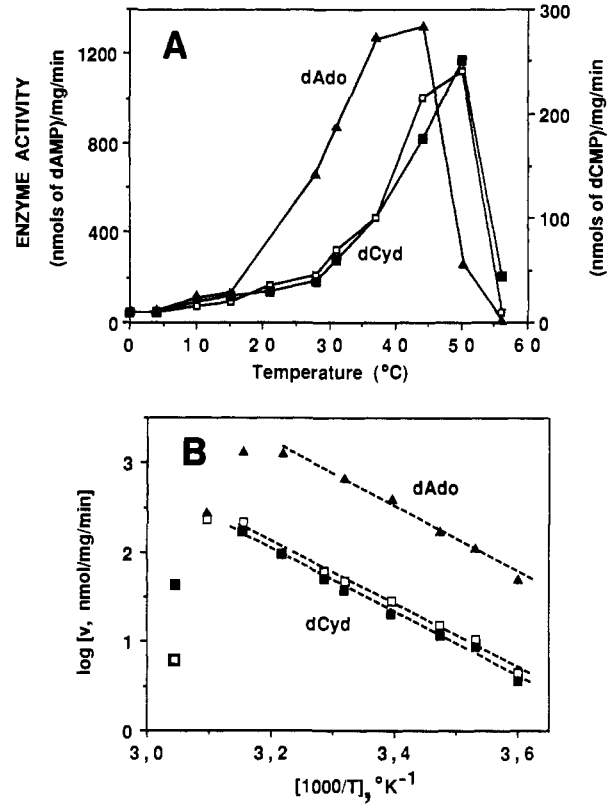

FIGURE 4: (A) Temperature dependence of the dCyd kinase activity toward 250 μM dAdo (▲) and dCyd at 5 μM (■) and 250 μM (□) as phosphate acceptors, with 1 mM ATP as phosphate donor. (B) An Arrhenius plot of the same data, where dashed lines represent a linear equation fitted to the linear part of the plots by using the linear regression method.

the effects of various detergents on the activity of the pure enzyme. We found that several detergents such as the Nonidet P-40 or Triton X-100 had very little effect but, unexpectedly, some more complex detergents like digitonin and CHAPS led to selective inhibition of dAdo activity without an effect on dCyd activity. The latter also had a stabilizing effect on the dAdo phosphorylating activity during incubation of the enzyme (see above). We chose to focus attention on three detergents, i.e., CHAPS, sodium cholate, and zwittergent 3-14 (Jones et al, 1986). The latter two are the components from which the complex detergent CHAPS is synthesized.

The effects of these detergents on kinase activities toward 10 μM dCyd and 200 μM dAdo, under standard assay conditions, are shown in Figure 5. There was a small stimulation of dCyd activity and a marked inhibition of dAdo activity observed with all three detergents in a concentration range of approximately 10% of the respective critical micelle concentration (cmc) for CHAPS and sodium cholate, and in the range of 50% of cmc for zwittergent 3-14. An Eadie–Hofstee plot of the dependence of dCyd kinase activity on dCyd concentration (Figure 6) showed a stimulation of the activity by CHAPS at high concentrations of dCyd. There was a change in the K_m values derived from the slopes, so that in the absence of CHAPS K_{m1} (the first) and K_{m2} (the second part of the curve) were 2 and 23 μM, respectively, and in the presence of detergent they were 1.2 and 13 μM, respectively. A Hill plot of the same data (Figure 6, insert) showed no clear-cut change in the dCyd-induced negative cooperativity of the enzyme. Furthermore, the substrate saturation curve of dCyd kinase with varying concentrations of dAdo (20–200 μM) at 1 mM ATP–$MgCl_2$ demonstrated that CHAPS (or sodium cholate) was a competitive inhibitor, and the double-reciprocal

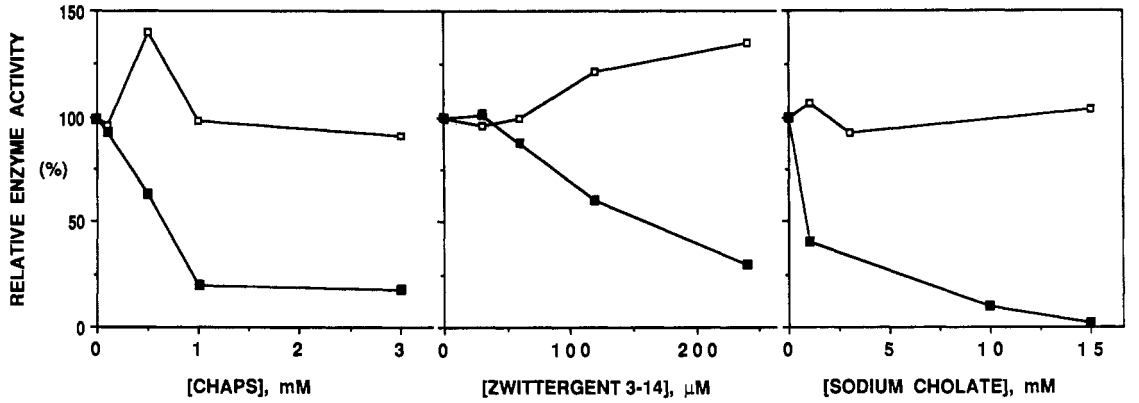

FIGURE 5: Effect of biological detergents, i.e., CHAPS, zwittergent 3-14, and sodium cholate, on the activity of pure dCyd kinase measured with 10 μM dCyd (□) or 100 μM dAdo (■) as phosphate acceptors and with 1 mM ATP as phosphate donor. Control values without detergents were 70 (with dCyd) and 490 nmol/(min·mg) (with dAdo), respectively.

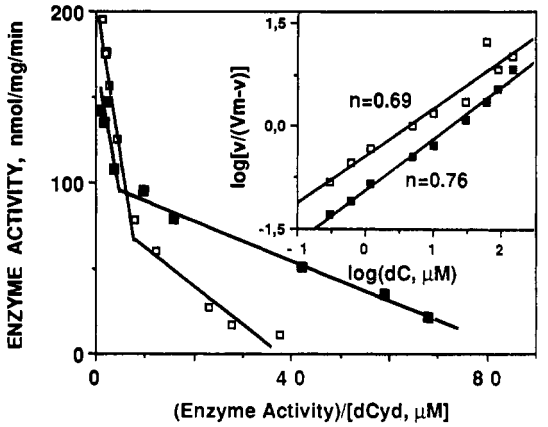

FIGURE 6: Eadie–Hofstee plot of the dependence of dCyd kinase activity on dCyd concentration in the absence (■) and in the presence of 0.5 mM CHAPS (□) with 1 mM ATP as phosphate donor. Insert shows a Hill plot of the same data and Hill constants (n = 0.69 ± 0.05 and n = 0.76 ± 0.03), respectively.

plot showed a characteristic pattern with a K_i value of 0.3 mM for CHAPS (data not shown).

The effect of 1.6 and 8.0 mM CHAPS on the overall subunit structure of the pure enzyme was determined with the aid of HPLC size-exclusion chromatography on a TSK-G3000SW column. The column was equilibrated and eluted with 50 mM Tris-HCl (pH 7.6) buffer containing 5 mM $MgCl_2$, 0.1 M KCl, and 1.6 or 8.0 mM CHAPS. The presence of CHAPS led to a decrease in the elution volume of both standard proteins and dCyd kinase by the same value, approximately 0.9 to 5% of the total volume of the column, respectively. The relative positions of standard proteins and dCyd kinase did not change, so the enzyme appeared to elute as a 60-kDa protein also in the presence of CHAPS. At 8.0 mM CHAPS, i.e., at its critical micelle concentration, the enzyme had no activity toward dAdo and its position was detected by the dCyd activity and SDS gel electrophoresis. These results show that the effect of CHAPS was not associated with alteration of the overall subunit structure of the enzyme.

Discussion

In this investigation we establish that the dCyd phosphorylating ability of pure dCyd kinase showed a different and higher stability than its dAdo activity. This was shown at various temperatures, and the most important stabilizing factors were the protein concentration and the presence of substrates or CHAPS. Inactivation occurred at different rates with different concentrations of dCyd kinase, even in the presence of a high concentration of BSA. Durham and Ives (1970b) also showed that calf thymus dCyd kinase was stabilized by addition of BSA, but they did not observe the selective inactivation described here.

The inactivation process was associated with an increased negative cooperativity when dCyd was used as substrate, while with dAdo (5–500 μM) there was an effect mainly on the V_{max}. We found no evidence for involvement of proteolysis in the inactivation process, nor was there a change in the overall enzyme structure; i.e., only the 60-kDa form was active with either dCyd or dAdo as substrates, as measured by gel filtration chromatography. Hence selective inactivation of dCyd kinase activity vs dAdo is not associated with gross alterations in enzyme structure.

We have measured the ratio of dAdo to dCyd activity in fractions during the last three purification steps (Bohman & Eriksson, 1988), i.e., DEAE-Sepharose, hydroxyapatite, and dTTP–Sepharose affinity chromatography, using 5 μM dCyd and 250 μM dAdo in the standard assay. This ratio was approximately 6 in all fractions, and thus the selective inactivation appeared to be minimal during these steps. It is worth noting that we took advantage of the stabilizing effect of dTTP during the dTTP affinity chromatography step. However, in the concentration and dialysis step following the dTTP chromatography we observed a selective inactivation of the dAdo activity especially with diluted protein solutions. Clearly, depending on the total amount of enzyme in the starting material and the conditions of enzyme purification and storage, large discrepancies in the state of the selective inactivation process may be obtained by different investigators. On the basis of results presented here, conditions were established that maintain the overall enzyme activity, i.e., by using stabilizing proteins like BSA, zwitterionic detergent CHAPS, and/or substrates. Methods to reactivate the enzyme with regard to its dAdo kinase activity have not yet been found.

The selective inactivation observed in this study is not unique; e.g., *Escherichia coli* DNA polymerase I also exhibits thermal-induced selective inactivation. At pH 8.5 and 42 °C, it lost 95% of its exonuclease activity, with retention of more than 50% of its polymerase activity. This phenomenon is reflected in a lower fidelity of replication (Lecomte & Doubleday, 1983). Another example is the potato tuber cyclic-nucleotide phosphodiesterase, where in alkaline pH at 45 °C it loses irreversibly and selectively the activity vs 3′,5′-cAMP with full maintenance of the activity vs 2′,3′-cAMP. There was also loss of about 80% of the activity vs thymidine 5′-(*p*-nitrophenyl phosphate) and thymidine 3′-(*p*-nitrophenyl phosphate) but retention of full activity vs *p*-nitrophenyl phenylphosphonate and bis(*p*-nitrophenyl) phosphate(Zan-

Kowalczewska et al., 1987). Selective inactivation of the latter proceeds at the level of the monomer, and as in our study, there is no indication of a connection between selective inactivation and changed subunit structure of the enzyme.

Our results show that the negative cooperativity with dCyd as substrate changes when the enzyme is selectively inactivated, but with only a minor effect on the V_{max} values derived from the slopes of the biphasic Eadie–Hofstee plots. It was earlier suggested that dCyd kinase may exist in two different conformational states: one responsible for phosphorylation of cytosine nucleosides and another form able to phosphorylate purine nucleosides (Bohman & Eriksson, 1988). The results presented here can be integrated into this model by postulating that the dCyd phosphorylating form of the enzyme is induced by dCyd and certain detergents and is more stable, while the other form is induced by the purine nucleosides and is less stable in the absence of substrates. The fact that there were two different temperature optima, one for the dCyd and other for the dAdo activity, respectively, is a strong indication that these two forms of dCyd kinase exist. Still earlier kinetic studies revealed that dCyd and dAdo were competitive inhibitors, presumably binding to the same active site but with very different K_m and K_i values. Recently, it was shown (Bohman et al., unpublished results) that both forms of the enzyme exhibit negative cooperativity since dCyd and dAdo saturation curves show this type of kinetics. The negative cooperativity is most likely related to the induction of different conformational forms of the enzyme, but the underlying mechanism remains to be clarified.

It is worth noting that the apparent activation energy of the dCyd and dAdo phosphorylation is similar for both low and high concentrations of dCyd, and it is not much different for the dAdo activity. They are also in the same range as that reported for thymidine kinase from *Escherichia coli* (Iwatsuki & Okazaki, 1967) and for many other enzymes [see Laidler and Peterman (1979) for review]. However, the interpretation of the apparent activation energy is not clear-cut because it is affected by many factors. It could include an activation energy of the rate constant for the rate-limiting step, the enthalpy changes for ionization and other equilibria in the enzyme reaction, or the activation energies for thermal conformational changes occurring in the protein molecule. At this stage an unequivocal interpretation of the activation parameters is not possible.

Detergents like CHAPS, sodium cholate, and zwittergent 3-14 were able to change the substrate specificity of dCyd kinase without changing the overall subunit structure. They inhibited effectively dAdo activity but gave a slight stimulation of the dCyd activity. To our knowledge, this is the first report of such a selective effect of a detergent on the substrate specificity of an enzyme. CHAPS appeared to act functionally as dCyd by inducing and stabilizing one form of the enzyme. It appears very likely that the detergent effect is mediated by direct binding to dCyd kinase and not to changes of the overall properties of the solution. However, since the inhibition was competitive with dAdo, there could be direct blocking of the detergent binding site(s) by dAdo. Alternatively, induction of the purine nucleoside conformational state of the enzyme by dAdo is able to overcome the inhibiting effect of the detergent.

Significant quenching of the protein fluorescence from dCyd kinase has been observed upon addition of dCyd and dAdo at concentrations equivalent to their respective K_m values (B. Kierdaszuk and S. Eriksson, unpublished results). Thus, direct binding studies can now be performed with dCyd kinase and its substrates. Results from these studies may provide definite answers to the nature of the selective inactivation process described here.

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