Identification of Purine Deoxyribonucleoside Kinases from Human Leukemia Cells: Substrate Activation by Purine and Pyrimidine Deoxyribonucleosides†

Jay C. Sarup‡,§ and Arnold Fridland*,‡,∥

Division of Biochemical and Clinical Pharmacology, St. Jude Children's Research Hospital, Memphis, Tennessee 38101, and Department of Pharmacology, The University of Tennessee, Memphis, Tennessee 38163

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ABSTRACT: Cell extracts from human leukemic T lymphoblasts and myeloblasts were chromatographed on DEAE-cellulose columns to separate purine deoxyribonucleoside, deoxyadenosine (dAdo) and deoxyguanosine (dGuo), phosphorylating activities. Three distinct purine deoxyribonucleoside kinases, a deoxycytidine (dCyd) kinase, an adenosine (Ado) kinase, and a deoxyguanosine (dGuo) kinase (the latter appears to be localized in mitochondria), were resolved. dCyd kinase contained the major phosphorylating activity for dAdo, dGuo, and 9-β-D-arabinofuranosyladenine (*ara*-A). Ado kinase represented a second kinase for dAdo and *ara*-A while a third kinase for dAdo was found in mitochondria. dCyd kinase was purified about 2000-fold with ion-exchange, affinity, and hydrophobic chromatographies. On gel electrophoresis, both dCyd and dAdo phosphorylating activities comigrated, indicating that the activities are associated with the same protein. The enzyme showed a broad pH optimum ranging from pH 6.5 to pH 9.5. Divalent cations Mg^{2+}, Mn^{2+}, and Ca^{2+} stimulated dCyd kinase activity; Mg^{2+} produced the maximal activity. dCyd kinase from either lymphoid or myeloid cells showed broad substrate specificity. The enzyme used several nucleoside triphosphates, but ATP, GTP, and dTTP were the best phosphate donors. dCyd was the best nucleoside substrate, since dCyd kinase had an apparent K_m of 0.3, 85, 90, and 1400 μM for dCyd, dAdo, dGuo, and *ara*-A, respectively. The enzyme exhibited substrate activation with both pyrimidine and purine deoxyribonucleosides, suggesting that there is more than one substrate binding site on the kinase. These studies show that, in lymphoblasts and myeloblasts, purine deoxyribonucleosides and their analogues are phosphorylated by dCyd kinase, Ado kinase, and dGuo kinase.

Purine deoxyribonucleosides dAdo[1] and dGuo and their metabolites have been implicated in the pathogenesis of inherited immunodeficiency diseases; these disorders accompany deficiencies of the catabolic enzymes adenosine deaminase and purine-nucleoside phosphorylase (Giblett et al., 1972, 1975). dAdo, dGuo, and the antileukemic analogue *ara*-A inhibit growth of cultured mammalian lymphocytes, T cells being more sensitive than B cells (Carson et al., 1982; Verhoef et al., 1981). Although the precise mechanisms of lymphocytotoxicity of purine deoxyribonucleosides are not fully understood, data indicate that cytotoxic effects of dAdo, dGuo, and *ara*-A are mediated primarily by their phosphorylated metabolites (Verhoef et al., 1981; Hershfield et al., 1982; Ullman et al., 1981).

Previous investigators using nucleoside kinase deficient cells demonstrated that two enzymes, Ado kinase (EC 2.7.1.20) and dCyd kinase (EC 2.7.1.74), phosphorylate dAdo and *ara*-A in intact T lymphoblasts (Verhoef et al., 1981; Hershfield et al., 1982). However, studies with partially purified nucleoside kinases suggest that there are several dAdo and dGuo phosphorylating activities in mammalian tissues. For example, purified Ado kinase from cultured ascites cells, mammalian liver, and brain (Lindberg et al., 1967; Miller et al., 1979; Yamada et al., 1980) but not from HEp-2 carcinoma or murine leukemia cells phosphorylates dAdo and *ara*-A (Schnebli et al., 1967; Chang et al., 1980). Similarly, partially purified dCyd kinase from calf thymus (Durham & Ives, 1970; Krenitsky et al., 1976) but not from human myeloid or murine leukemia cell phosphorylates dAdo and dGuo (Coleman et al., 1975; Cheng et al., 1977; Brockman et al., 1980). In agreement with this, Meyers and Kreis (1976) chromatographically separated dGuo–dAdo phosphorylating activity from dCyd kinase using murine leukemia cell extracts. Additionally, a dGuo-specific kinase has been isolated from calf thymus mitochondria (Gower et al., 1979) and from mouse skins (Barker & Lewis, 1981).

The purpose of this study was to identify, to purify, and to characterize purine deoxyribonucleoside (dAdo and dGuo) phosphorylating activities in human lymphoid and myeloid cells. We report here that human T lymphoblasts and myeloblasts contain at least three distinct purine deoxyribonucleoside phosphorylating activities. dCyd kinase, which is the major dAdo, dGuo, and *ara*-A phosphorylating activity,

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* Author to whom correspondence should be addressed at St. Jude Children's Research Hospital.

‡ The University of Tennessee.

§ Present address: Department of Microbiology and Molecular Biology, University of California, Los Angeles, CA 90024.

∥ St. Jude Children's Research Hospital.

[1] Abbreviations: dCyd, deoxycytidine; dAdo, deoxyadenosine; dGuo, deoxyguanosine; *ara*-A, 9-β-D-arabinofuranosyladenine; *ara*-C, 1-β-D-arabinofuranosylcytosine; DTT, dithiothreitol; CEM, CCRF-CEM (human leukemic T lymphoblasts termed CEM); CML, chronic myelogenous leukemia; T-ALL, T acute lymphoblastic leukemia; dThd, thymidine; DEAE-cellulose, (diethylaminoethyl)cellulose; ATP, adenosine 5′-triphosphate; GTP, guanosine 5′-triphosphate; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; dTTP, thymidine 5′-triphosphate; dCF, deoxycoformycin; BSA, bovine serum albumin; dIno, deoxyinosine.

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was purified about 2000-fold and characterized with both purine ad pyrimidine deoxyribonucleosides as substrates.

Materials and Methods

Chemicals and Enzymes. Nucleosides, nucleotides, phosphoenolpyruvate (monocyclohexylammonium salt), Hepes, Tris base, rabbit muscle pyruvate kinase type II, milk xanthine oxidase, grade V chicken egg albumin, and Cohn fraction V bovine serum albumin were purchased from Sigma Chemical Co. (St. Louis, MO). Yeast alcohol dehydrogenase and pig heart mitochondrial malate dehydrogenase were from Worthington Biochemicals Corp. (Freehold, NJ). Dithiothreitol was from Calbiochem (San Diego, CA). Glycerol was from J. T. Baker Chemical Co. (Phillipsburg, NJ), and ultrapure sucrose was from Schwarz/Mann (Orangeburg, NY). Unlabeled *ara*-A and deoxycoformycin were from the Drug Development Branch of the National Cancer Institute (Bethesda, MD). Unlabeled *ara*-C was purchased from the Upjohn Co. (Kalamazoo, MI). *N,N'*-Methylenebis(acrylamide) was from Eastman Kodak Co. (Rochester, NY), acrylamide was from Bio-Rad Laboratories (Richmond, CA), and *N,N,N',N'*-tetramethylethylenediamine was from BDH Chemicals (Poole, England). DEAE-cellulose DE-23 and DE-81 discs were from Whatman Inc. (Clifton, NJ). Hexylagarose was a gift from Dr. R. L. Blakley (St. Jude Children's Research Hospital, Memphis, TN). [U-^{14}C]Cytidine (0.485 Ci/mmol), [U-^{14}C]deoxyguanosine 5'-monophosphate (0.482 Ci/mmol), and [8-^{3}H]deoxycytidine (19.3 Ci/mmol) were obtained from Amersham (Arlington Heights, IL). [5,6-^{3}H]-*ara*-C (18 Ci/mmol) and [8-^{3}H]deoxyadenosine (20 Ci/mmol) were from Moravek Biochemicals (City of Industry, CA), and [2-^{3}H]adenosine (20 Ci/mmol) was from Schwarz/Mann (Orangeburg, NY). All other reagents were of the highest quality generally obtainable.

Cells and Cell Culture. Leukophoresed cells were obtained from patients admitted to St. Jude Children's Research Hospital, Memphis, TN. Cells from patients with acute T-lymphoblastic leukemia (T-ALL) were positive for E rosette formation with sheep erythrocytes, and cells from patients with chronic myelocytic leukemia (CML) in blast crisis were positive for the Philadelphia chromosome; these diagnostic reports were provided by physicians at the St. Jude Children's Research Hospital. CCRF-CEM (human leukemic T lymphoblasts termed CEM) were cultured routinely as described previously (Verhoef et al., 1981).

Enzyme Assays. Phosphorylating activities for all nucleosides were determined by the DE-81 disc method of Ives et al. (1969), modified as described below. Adenosine kinase was assayed according to the method of Verhoef et al. (1981), except that 2 μM dCF was used as adenosine deaminase inhibitor. Unless otherwise stated, phosphorylation activities for all other nucleosides were determined at 37 °C in a final volume of 50–200 μL of a reaction mixture containing 0.1 M Hepes (pH 7.5), 3 mM DTT, 10 mM ATP, 10 mM $MgCl_2$, 10 mM NaF, 15 mM phosphoenolpyruvate, 5 unit s/mL pyruvate kinase, and appropriate radioactive nucleoside: [^{3}H]dCyd (80 μM, 62.5 Ci/mol), [^{3}H]-*ara*-C (200 μM, 50 Ci/mol), [^{3}H]dAdo (1.0 mM, 10 Ci/mol), [^{3}H]dGuo (1.0 mM, 10 Ci/mol), or [^{3}H]*ara*-A (1.0 mM, 10 Ci/mol). In kinase assays with dAdo and *ara*-A, 2 μM dCF was also present. In assays with either blue Sepharose- or hexylagarose-purified dCyd kinase, 0.5 mg/mL BSA was included in the reaction mixture, but the following were omitted: phosphoenolpyruvate, pyruvate kinase, dCF, and NaF. The reaction mixture was incubated for 5 min, and then the kinase reactions were initiated by the addition of enzyme. The reactions were stopped by 5-fold dilution of the reaction mixtures with water, and then immediately 50 μL of the diluted mixture was applied per DE-81 disc. The discs were dried at room temperature, washed with water, and dried at 60 °C, and then the discs were placed in scintillation vials containing 1.0 mL of 0.2 M KCl in 0.1 N HCl for 5 min to elute nucleotide products (Ives et al., 1969). The samples were quantified for radioactivity in 7.0 mL of aqueous counting scintillation fluid (ACS, Amersham). Nonspecific binding to DE-81 discs, determined from kinase reaction mixtures without enzyme, was less than 2%, and this value was subtracted from the samples with enzyme.

Linearity with time and protein concentration was observed with all enzyme assays. Linearity with time for all assays was established for the different substrate concentrations used in initial velocity studies.

An appropriate amount of enzyme was chosen so that, at the lowest substrate concentration to be studied, no more than 30% of the substrate was converted to product. The kinase reactions were linear through this extent of complication. The amount of nucleoside monophosphate formed per 30 or 45 min at each concentration of a variable substrate represented initial velocity.

5'-Nucleotidase activity was determined with [^{14}C]dGMP as the substrate by the method of Ives et al. (1969). Cytidine deaminase was assayed according to Verhoef et al. (1981). Adenosine deaminase, purine-nucleoside phosphorylase, and alcohol dehydrogenase activities were determined by the methods of Agarwal et al. (1975), Kim et al. (1968), and Vallee and Hoch (1955), respectively. Malate dehydrogenase was assayed spectrophotometrically at 22 °C by measuring the decrease in absorbance at 340 nm in a reaction mixture containing 0.1 M potassium phosphate buffer (pH 7.4), 0.25 mM NADH, 0.2 mM oxaloacetate, and the enzyme (Worthington Enzyme Manual, Worthington Biochemical Corp., Freehold, NJ).

Unit of Enzyme Activity. One unit represents the amount of enzyme required to convert 1 nmol of substrate to product per 1 h under the assay conditions.

Mitochondrial Extract. Mitochondria were isolated from about 20 × 10^{9} CEM or T-ALL cells on the basis of the procedure of Johnson and Lardy (1967), except that the cells were suspended at 2 × 10^{8} cells/mL of Hepes-buffered sucrose [10 mM Hepes (pH 7.5) and 0.25 M sucrose] and disrupted in a Potter–Elvehjem homogenizer by applying 25 strokes manually. The mitochondrial pellet was washed twice with 10 mL of Hepes-buffered sucrose and then resuspended in 15 mL of 10 mM Hepes (pH 7.5) containing 3 mM DTT. The suspension was sonicated for 2 min over ice and then centrifuged at 100000*g* for 60 min. The supernatant (mitochondrial extract) was removed and stored at −20 °C for enzyme assays. Citrate synthase, which is localized exclusively in mitochondria (Srere, 1969) was assayed by the method of Srere (1969) to confirm the presence of mitochondrial enzymes in the extracts. The presumptive mitochondrial extracts, prepared as above, contained about 70% of total cellular citrate synthase activity but no adenosine kinase, a cytosolic enzyme, thus confirming that the extract is of mitochondria.

Enzyme Purification. (A) Cell Extract. For routine use, about 0.4 mL of cell extract was made from 1 × 10^{8} CEM cells. All the steps in the preparation of cell extracts and enzyme purification were done at 4 °C. Cells were collected by centrifugation at 500*g* for 10 min and then washed twice with 10 cell pellet volumes of phosphate-buffered saline [0.9% NaCl, 10 mM K_2HPO_4/KH_2PO_4 (pH 7.2)]. The washed cells

were resuspended at 2×10^8 cells/mL of homogenization buffer [10 mM Hepes (pH 7.2) and 3 mM DTT] and homogenized manually in a Potter–Elvehjem homogenizer with 20 strokes. The homogenate was centrifuged at 100000*g* for 60 min, and the supernatant (cell extract), without dialysis, was used for enzyme studies.

For the purpose of enzyme purification, cell extracts were prepared by sonication as described below. The patient (T-ALL or CML) cells, which had about 5% contamination with red blood cells, were allowed to settle in graduated cylinders at 4 °C for 3 h. Red cells formed the bottom layer, and the layer above, composed of lymphocytes or myeloblasts, was siphoned and saved for further processing. The cells were washed as described above, and then CEM cells were resuspended at 2×10^8 cells/mL of homogenizer buffer, whereas patient cells were resuspended at 4×10^8 cells/mL of homogenization buffer. The cell suspensions, in 100-mL aliquots, were sonicated for 3 min. The cell homogenates were centrifuged at 100000*g* for 60 min, and the supernatant was removed and dialyzed against 10 volumes of buffer A [50 mM Tris-HCl (pH 7.5) and 3 mM DTT] for 36 h with three changes of the buffer.

(*B*) *DEAE-cellulose Chromatography.* Ion-exchange chromatography was performed in a column with precycled Whatman DE-23 cellulose equilibrated with buffer. The protein sample, diluted to an appropriate volume with equilibration buffer, was applied to the column and unbound material collected in a batch. The column was then eluted with a linear gradient from 0 to 1.0 M KCl in equilibration buffer. The 0–1.0 M KCl gradient was collected in 2-mL fractions. Every second fraction was assayed for adenosine kinase, deoxyadenosine kinase, deoxyguanosine kinase, and deoxycytidine kinase.

(*C*) *Blue Sepharose Chromatography.* The DEAE-cellulose-purified dCyd kinase (peak I) from either T-ALL or CEM cells was concentrated and dialyzed against 50 mM Tris-HCl (pH 7.5), 3 mM DTT, 5 mM $MgCl_2$, and 10% glycerol (buffer B) by the Amicon YM-10 concentrator. The enzyme was then applied to a blue Sepharose column (2×8.5 cm) prepared according to Bohme et al. (1972) equilibrated with buffer B. The column was then washed with buffer B until the absorbance at 280 nm returned to base-line level. dCyd kinase was eluted with 150 mL of a linear 0–10 mM NADH gradient in buffer B. Fractions (3 mL) were collected and assayed for kinase activity for dCyd, dAdo, and dGuo. The enzyme fractions were concentrated to 1.0 mg/mL and applied to a second blue Sepharose column (2×6 cm), equilibrated with buffer B. The column was washed with buffer B to remove unbound material and then eluted with 90 mL of a linear 0–7 nM ATP–$MgCl_2$ gradient and then 30 mL of 7 mM ATP–$MgCl_2$, both made in buffer B. Fractions containing the peak of dCyd–dAdo kinase activity were pooled, concentrated 50-fold, dialyzed in buffer A containing 10% glycerol, and then concentrated to 1.0 mg/mL with an Amicon YM-10 concentrator.

(*D*) *Hexylagarose Chromatography.* Hexylagarose was washed according to the manufacturer's directions and equilibrated with 50 mM Tris-HCl (pH 7.5), 5 mM DTT, 1 mM $MgCl_2$, 1 mM ATP, 0.75 M $(NH_4)_2SO_4$, and 10% glycerol (buffer C). A dialyzed sample of partially purified dCyd–dAdo kinase was applied to the column (1.0×1 cm) of phenylagarose. The column was washed with buffer C to remove unbound material, and then 75 mL of a linear decreasing 0.75–0 M $(NH_4)_4SO_4$ gradient in 50 mM Tris-HCl (pH 7.5), 5 mM DTT, 1 mM $MgCl_2$, 1 mM ATP, and 10% glycerol (buffer D) was applied to the column to elute the kinase activities. Three-milliliter fractions were collected into tubes containing glycerol (20% final concentration). Fractions containing the deoxynucleoside phosphorylating activities were pooled, desalted, and concentrated to 0.1 mg/mL in 50 mM Tris-HCl (pH 7.5) containing 5 mM DTT, 0.25 mM ATP, 0.25 mM $MgCl_2$, and 50% glycerol.

(*E*) *Gel Electrophoresis.* Analytical polyacrylamide gel electrophoresis was performed with a modification of the method of Gabriel (1971). Kinases were electrophoresed at 4 °C on polyacrylamide slab gels made up of a 3.5% stacking gel (1.5×15 mm) and a 7.5% separating gel (1.5×80 mm). The gels were made and run in a continuous buffer [0.1 M Tris-HCl (pH 8.5 at 4 °C) and 1 mM DTT]. Bromophenol blue (0.005%), applied to a separate lane, served as the tracking dye. After the samples were loaded, 15 mA per gel was applied until the dye penetrated the separating gel, and then 30 mA per gel was applied until the dye reached the bottom of the gel. The lanes to be assayed for enzyme activity were sliced into 4-mm pieces, and then each piece was incubated in 150 μL of a kinase reaction mixture at 37 °C for 6 h and the amount of product formed determined under the standard procedures. The other lanes were stained with a silver stain kit (Bio-Rad Laboratories, Richmond, CA) and scanned by an Ortec 4310 photon-counting densitometer. Integration of peak areas was done by an Ortec 6240B multichannel analyzer and a Hewlett-Packard 9845T computer.

(*F*) *Protein Determination.* Protein concentrations were determined with a Bio-Rad protein assay kit with bovine γ-globulin as the standard (Bio-Rad Laboratories, Richmond, CA).

RESULTS

Separation of Identification of Purine Deoxyribonucleoside Phosphorylating Activities. Cell extracts from lymphoblasts (CEM and T-ALL) and myeloblasts (CML) were chromatographed on DEAE-cellulose columns to separate purine deoxyribonucleoside (dAdo and dGuo) phosphorylating activities. Three peaks of purine deoxyribonucleoside phosphorylating activities were found for CEM cell extracts. The major peak of dAdo, dGuo, and *ara*-A phosphorylating activities coeluted with dCyd kinase (Figure 1A, peak I). About 60, 36, 40, and 50% of dCyd, dAdo, *ara*-A, and dGuo phosphorylating activities, respectively, present in cell extracts, were recovered in peak 1. A second peak of dAdo and *ara*-A phosphorylating activity coeluted with Ado kinase (Figure 1A, peak 2). About 90, 15, and 10% of Ado, dAdo, and *ara*-A phosphorylating activities, respectively, present in cell extracts, were recovered in peak 2. There was no dGuo phosphorylating activity associated with Ado kinase. A third and smaller peak of kinase(s) (Figure 1A, peak 3) that phosphorylated dGuo, dAdo, and dCyd eluted at about 0.3 M KCl. Peak 3 contained less than 1% of dGuo, dAdo, or dCyd phosphorylating activities present in cell extracts. These dGuo, dAdo, and dCyd kinase activities (peak 3) appear to be of mitochondrial origin and will be described in a later section. Results similar to those of CEM cells were obtained by chromatography of T-ALL cell extracts, except that Ado kinase (peak 2) had no purine deoxyribonucleoside phosphorylating activity (Figure 1B, peak 2). Hurley et al. (1983) reported that potassium ion was necessary for dAdo phosphorylation by the purified placental Ado kinase. When T-ALL Ado kinase was assayed with dAdo (50 μM) in the presence of 30 mM KCl, the enzyme phosphorylated dAdo at a rate of 5 nmol of dAMP h^{-1} (mg of protein)$^{-1}$, and thus KCl restored purine deoxyribonucleoside phosphorylating activity to the T-ALL Ado kinase. Although

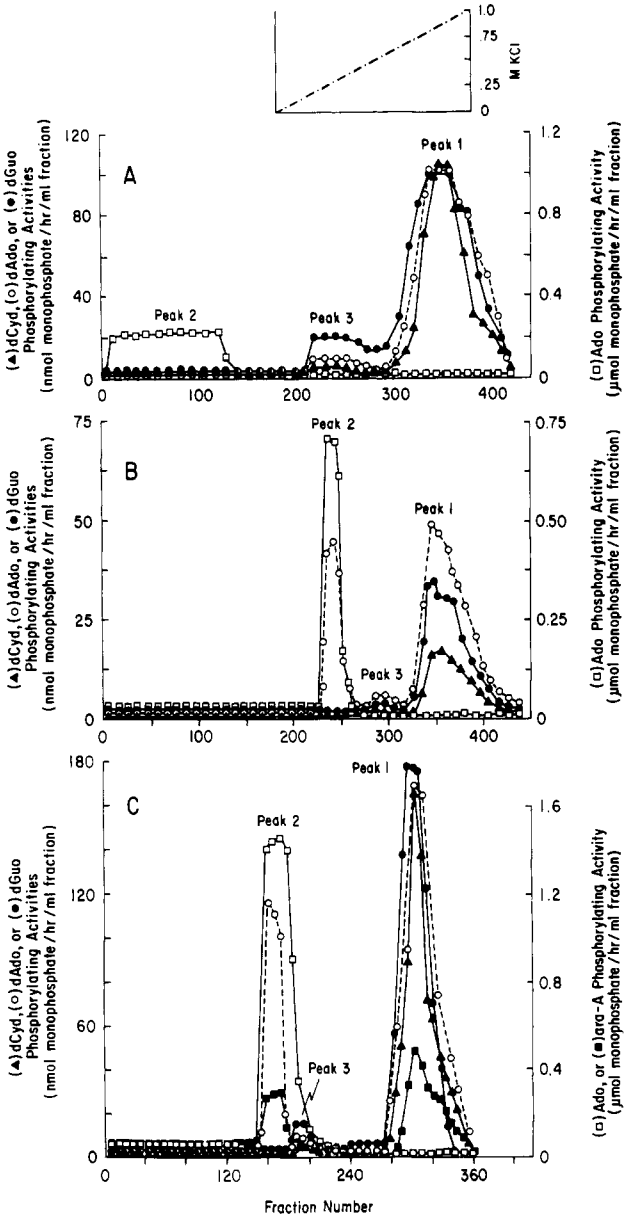

FIGURE 1: Resolution of purine deoxyribonucleoside phosphorylating activities in lymphoid and myeloid cell extracts by DEAE-cellulose chromatography. Cell extracts from CEM (60×10^9 cells), T-ALL (50×10^9 cells), and CML (345×10^9 cells) were applied to DEAE-cellulose columns, which were 2.9 × 45 cm for CEM and T-ALL and 9.0 × 18.5 cm for CML cells. The columns were washed with buffer A to remove unbound protein, and then a linear 0–1.0 M KCl gradient was applied to the columns to elute the kinases as detailed under Materials and Methods. Fractions, 3 mL for CEM and T-ALL and 2 mL for CML columns, were collected. Aliquots of the fractions were assayed for dCyd (80 μM; ▲), dAdo (50 μM; ○), dGuo (50 μM; ●), *ara*-A (1.0 mM; ■), and Ado (100 μM; □) phosphorylating activities. Panels A–C are elution profiles for CEM, T-ALL, and CML cell extracts, respectively. Kinase activities for dCyd, dAdo, and dGuo are in nmol of monophosphate formed h^{-1} (mL of fraction)$^{-1}$, whereas that for Ado is in μmol of monophosphate formed h^{-1} (mL of fraction)$^{-1}$.

CEM Ado kinase phosphorylated dAdo in the absence of potassium ion, 30 mM KCl stimulated dAdo (50 μM) phosphorylation by about 3-fold. The elution profiles and the identity of purine deoxyribonucleoside phosphorylating activities from nonlymphoid (CML) cells were the same as those observed with the lymphoid (CEM) cells (Figure 1C). These results show that there are at least three kinases, dCyd kinase, Ado kinase, and dGuo/dAdo/dCyd kinase(s), for phosphorylation of the purine deoxyribonucleosides.

Table I: Kinetic Parameters for Substrates of Ado Kinase[a]

substrate	K_m^{app} (μM)	V_{max}^{app} (nmol h^{-1} mg^{-1})	relative efficiency (V_{max}/K_m)
Ado	2.3	3989	100
dAdo	741 (2600)	173 (74)	0.014 (0.0016)
ara-A	3590 (12600)	69 (23)	0.0011 (0.0001)

[a] The kinetic constants were calculated from Lineweaver–Burk plots, and the methods for calculation of the enzyme efficiency are given under Materials and Methods. The concentration of ATP and Mg was 10 mM, and the enzyme activity was assayed at pH 6.0 for Ado and at pH 6.0 (in parentheses) and pH 7.5 for dAdo and *ara*-A.

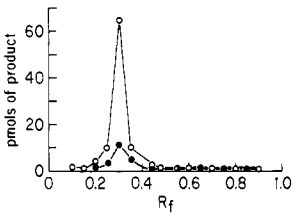

FIGURE 2: Deoxyguanosine kinase activity assayed following polyacrylamide gel electrophoresis of mitochondria extract (○) and peak 3 (●) from DEAE-cellulose column. The R_f shown refers to the mobility relative to bromophenol blue. The gel was sliced into 4-mm pieces and assayed with 50 μM dGuo in 150 μL of kinase reaction mixture as described under Materials and Methods.

Substrate Specificity of Ado Kinase (Peak 2). Specificity of Ado kinase toward Ado, dAdo, and *ara*-A was studied with 10-fold DEAE-cellulose-purified CEM Ado kinase (Figure 1C). Ado was the best substrate for Ado kinase as seen by the lowest K_m and highest V_{max} (Table I). Ado kinase showed about a 10-fold lower efficiency with *ara*-A than with dAdo. When dAdo and *ara*-A kinetics were done in the Ado kinase reaction mixture at pH 6, the K_m increased and the V_{max} decreased from the values obtained at pH 7.5. The change in pH also caused a 10-fold decrease in the efficiency of the enzyme with dAdo and *ara*-A (Table I, kinetic constants in parentheses), indicating that dAdo and *ara*-A are better substrates for Ado kinase at neutral pH. Ado kinase did not phosphorylate dGuo (1.0 mM) or dCyd (80 μM) at 0.75 mg of Ado kinase protein per reaction mixture. This enzyme concentration was 10-fold greater than needed for phosphorylation of *ara*-A, the poorest substrate. These studies demonstrate that Ado kinase is restricted toward purine nucleosides with an adenine ring, such as Ado, dAdo, or *ara*-A.

Peak 3 and Mitochondrial Deoxynucleoside Kinases. Since the kinase activity for dGuo (Figure 1) eluted with 0.3 M KCl from DEAE-cellulose columns, similar to the calf thymus mitochondrial dGuo kinase (Gower et al., 1979), we have separated cell extracts into mitochondrial and cytoplasmic fractions in order to ascertain the subcellular location of peak 3 enzyme. Extracts of mitochondria contained phosphorylating activities toward the four deoxynucleosides dGuo, dAdo, dCyd, and dThd. The specific activities of the kinases with dGuo, dAdo, dCyd, and dThd as substrates at 50 μM were 7, 4, 1, and 3 units per mg of protein, respectively. The mitochondrial fraction was essentially free of adenosine deaminase activity and converted dAdo mostly to dAMP. Therefore, dAdo phosphorylating activity in human mitochondrial extract is not due to the formation of dIno, which is a substrate for calf thymus mitochondrial dGuo kinase (Gower et al., 1979). Extract from the mitochondria and fractions from peak 3 of the DEAE-cellulose fractions were also subjected to gel electrophoresis. The dGuo kinase activities in mitochondrial extracts and in peak 3 showed similar electrophoretic mobilities with R_f values of 0.25 (Figure 2). By contrast, the dGuo phosphorylating activity in peak 1 of the DEAE-cellulose

Table II: Purification Summary of dCyd Kinase from T-ALL Cells[a]

purification step	total protein (mg)	total act. (units)	sp act. (units/ protein)	yield (%)	x-fold purification	dCyd:dAdo ratio
cell extract	2406	3510	1.5	100	1.0	0.19
DEAE-cellulose	115	4530	30	130	21	0.09
blue Sepharose I	8.75	2800	320	80	220	0.08
blue Sepharose II	2.90	1780	610	50	420	0.10
hexylagarose	0.13	360	2700	10	1850	0.11

[a] One unit equals 1 nmol of dCMP formed per 1 h under standard assay conditions. The ratio of dCyd phosphorylation to dAdo phosphorylation was calculated from the respective specific activity.

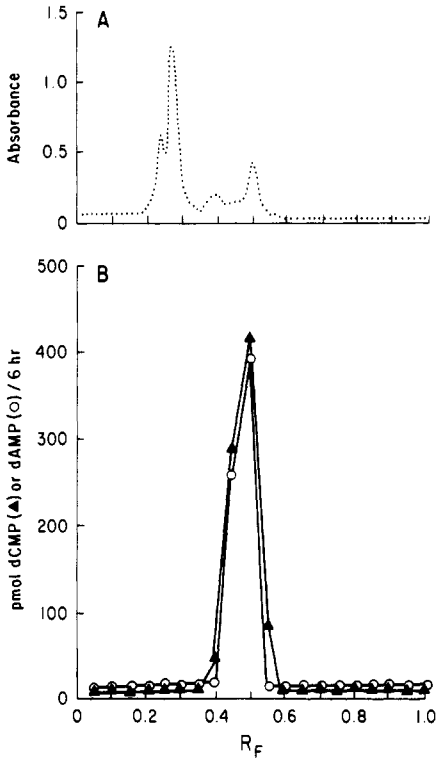

FIGURE 3: Discontinuous polyacrylamide gel electrophoresis of hexylagarose-purified enzyme (20 μg) loaded onto 7.5% nondenaturating polyacrylamide gels and at the end of the run, the gel being scanned with a densitometer and assayed for enzyme activity. The R_f shown in (B) refers to the mobility relative to bromophenol blue. For determination of enzyme activity, the gel was sliced into 4-mm pieces, and the slices were assayed either with 5 μM dCyd (△) or with 20 μM dAdo (○) in 150 μL of kinase reaction mixture as described under Materials and Methods.

fractions migrated solely with R_f 0.55 as in Figure 3.

Phosphorylating activity for dAdo (50 μM) in peak 3 or in mitochondrial extract was inhibited approximately 70% by unlabeled dGuo, suggesting a common kinase for the two nucleosides (data not shown). Unlike peak 1 enzyme, dCyd did not inhibit dGuo or dAdo phosphorylations by peak 3 or by mitochondrial extract. These results are consistent with the view that the two protein fractions are entirely distinct and derived from cytosol and mitochondria, respectively.

Characterization of Deoxycytidine Kinase. (A) Purification. dCyd kinase was purified about 2000-fold with at least 10% yield by a combination of ion-exchange, affinity, and hydrophobic chromatographies (Table II) from both the T-ALL and CEM blasts. Purification of dCyd kinase in the absence of DTT and glycerol resulted in about 3-fold lower activity than in their presence. Mercaptoethanol (10 mM) was not effective in stabilizing dCyd kinase during purification. During the purification, the ratio between the activities of the three substrates dCyd, dAdo, and dGuo remained roughly constant, and no indication for a separation of different deoxynucleoside kinase activity was found after the DEAE-cellulose step (Table II). The final enzyme preparation was assayed for the phosphorylation of various other nucleosides, including the arabinonucleosides of adenine, guanine, and cytosine. The maximal activity for these analogues was 3, 7, and 31%, respectively, of their natural deoxynucleoside counterpart. Other nucleosides, including cytidine, adenosine, uridine, and thymidine, were not phosphorylated. Human enzyme from T-ALL blasts was tested for purity after separation by nondenaturing gel electrophoresis. The profile in Figure 3 shows the congruence of dCyd and dAdo kinase activities; a similar profile was obtained for dGuo phosphorylating activity and with varying gel concentrations (data not shown). Four protein bands were observed following silver staining of the polyacrylamide gel. The main protein band did not correspond to the deoxynucleoside kinase activities as determined by enzyme activity after elution from the gel (Figure 3B).

(B) Test for Auxiliary Reactions. Measurement of substrate specificity could be affected by enzymes other than nucleoside kinase, which may be present in the enzyme preparation and which acts on the substrates or products. The following activities were compared to the activities of the corresponding deoxynucleoside kinases. The final enzyme preparation contained adenosine deaminase (<2%), purine-nucleoside phosphorylase (<3%), cytidine deaminase (<1%), 5′-deoxynucleotidase (<2%), and nucleoside monophosphate kinase (<5%). Therefore, assays of the purified kinase activity were not significantly altered by reactions competing for the substrates or products.

(C) Stability, Storage Conditions, and Effects of BSA and DTT. dCyd kinase purified through the second blue Sepharose column was stable for about 2 years when stored at −20 °C at a protein concentration of 1.7 mg/mL in 50% glycerol containing 50 mM Tris-HCl (pH 7.5), 5 mM DTT, 0.25 mM ATP, and 0.25 mM $MgCl_2$. Hexylagarose-purified dCyd kinase (0.1 mg of protein/mL) was considerably less stable, since it lost about 70% of dCyd and dAdo phosphorylating activities in 6 weeks. This rapid loss of the activity is possibly due to storage of the enzyme at low protein concentration. BSA (0.25 mg/mL) had no effect on the activity of blue Sepharose purified dCyd kinase. However, BSA caused a slight (1.3-fold) stimulation of hexylagarose-purified dCyd kinase activity. DTT (3 mM) increased dCyd kinase activity by about 2-fold in the partially purified preparations but not in cell extracts.

(D) pH Optimum and Metal Ion Requirements. The optimal pH for dCyd kinase activity was determined with both dCyd and dAdo as substrates in Hepes (pH 6.5–8.0) and Tris-HCl (pH 8.0–9.0) buffers. dCyd kinase showed a broad pH optimum, 6.5–9.0 for T-ALL and CEM and 7.5–9.0 for CML enzyme, with either dCyd or dAdo as substrates. In the absence of added divalent cations, dCyd is phosphorylated at about 45% the rate observed with the optimal $MgCl_2$

Table III: Phosphate Donor Specificity for T-ALL Deoxycytidine Kinase

nucleoside triphosphate (0.1 mM)	relative activity[a]			
	dCyd	dAdo	dGuo	*ara*-A
ATP	100 (191)	100 (370)	100 (122)	100 (2840)
dATP	52	0	21	0
GTP	120	108	63	129
dGTP	106	30	33	32
dTTP	94	90	71	67
UTP	27	44	39	20
CTP	45	0	0	0
dCTP	0	0	0	0

[a] Kinase activity obtained with ATP is taken as 100, and kinase activities with other nucleotides are relative to the ATP value. The number in parentheses is picomoles of product formed during the incubation with ATP as the phosphate donor.

concentration while that of dAdo or dGuo phosphorylation is about 15% of the control rate. Mn^{2+} and Ca^{2+} were 70 and 30%, respectively, as efficient as Mg^{2+}. At 2.0 mM ATP, the optimal ATP:Mg ratios were 1:1 with dCyd and 1:5 with dAdo as substrates. Free Mg^{2+}, at least 48 mM in excess over ATP, was not inhibitory to dCyd or dAdo phosphorylating activities of dCyd kinase.

(*E*) *Substrate Specificity of dCyd Kinase.* Deoxycytidine kinase was characterized with respect to both phosphate donor and acceptor substrates. The ability of dCyd kinase to utilize a triphosphate was partly dependent on the phosphate acceptor; however, ATP and GTP were the best phosphate donors for all four substrates dCyd, dAdo, dGuo, and *ara*-A (Table III). With dCyd as the substrate, dCyd kinase utilized a broad range of triphosphates including ATP, GTP, dGTP, dTTP, and dATP; the former four nucleotides were about equally effective. dGTP was a poor phosphate donor when purine nucleosides were acceptor substrates. dCTP did not serve as phosphate donor for dCyd kinase at a detectable rate.

Nucleoside substrate specificity of dCyd kinase was assessed by comparison of kinetic parameters K_m and V_{max} determined over a wide range of nucleoside concentrations and at a saturating level (10 mM) of ATP. The Lineweaver–Burk plots of T-ALL dCyd kinase (hexylagarose purified) with dCyd, dAdo, and dGuo showed bimodal saturation; this result implies substrate activation (Figure 4A–C). In contrast, the enzyme showed Michaelis–Menten kinetics with the analogue *ara*-A up to 4.0 mM, the highest concentration tested (Figure 4D). dCyd kinase of lower degree of purity, DEAE-cellulose purified, also showed substrate activation with dCyd, dAdo, dGuo, *ara*-C, and ATP but not with *ara*-A (data not shown). dCyd kinase from CEM and myeloid cells also showed bimodal saturation with dCyd, dAdo, dGuo, *ara*-C, and ATP but not with *ara*-A. This non-Michaelis–Menten kinetics with either dCyd or dAdo as varied substrate was observed at nonsaturating (0.5 mM) and saturating (10 mM) ATP and in the absence or presence of either BSA or DTT.

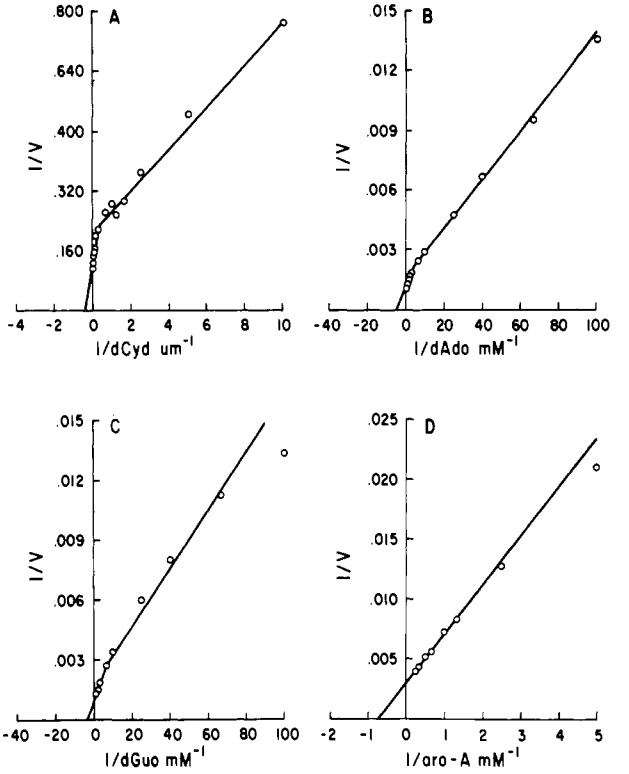

FIGURE 4: Lineweaver–Burk plots of dCyd kinase activity with nucleosides as the variable substrate. Initial velocities (V) were determined by varying dCyd (A), dAdo (B), dGuo (C), or *ara*-A (D) concentrations and by using hexylagarose-purified T-ALL dCyd kinase at fixed concentration of ATP and MgCl in a 5-fold excess of ATP. Reaction volume was 50 μL, and the amount of enzyme protein was 50 ng in (A) and 250 ng in (B–D). Velocities are in pmol of nucleoside monophosphate formed/30 min.

dCyd kinase from either lymphoid or myeloid cells showed greatest affinity for dCyd, since K_m values for this substrate, 0.3–0.4 μM, were about 100–200-fold lower than for dAdo and dGuo (Tables IV and V). Purification of dCyd kinase from DEAE-cellulose through hexylagarose steps did not affect the K_m values of the enzyme for its substrates (data not shown). Although dCyd was the best substrate when assessed by K_m values, substrates with higher K_m gave higher V_{max}. Therefore, specificity of dCyd kinase was determined from the ratio of V_{max} to K_m, termed catalytic efficiency (Krenitsky et al., 1976; Cornish-Bowden, 1979). dCyd kinase from either lymphoid or myeloid cells showed about 10-fold greater catalytic efficiency with dCyd than with dAdo or dGuo (Tables IV and V). *ara*-A was the poorest substrate, being 10^{-3}–10^{-5} as efficient as dCyd. Comparison of catalytic efficiencies (Tables IV and V) shows that dCyd kinase prefers cytosine over adenine and the deoxyribose sugar moiety over arabinose or the ribose. These results demonstrate that dCyd kinase from lymphoid cells and dCyd kinase from myeloid cells are similar

Table IV: Kinetic Parameters for T-ALL dCyd Kinase[a]

substrate	K_m^{app} (μM)[b]		V_{max}^{app} (nmol h^{-1} mg^{-1})[b]		relative efficiency[c]	
	$K_{m,1}$	$K_{m,2}$	$V_{max,1}$	$V_{max,2}$	$V_{max,1}/K_{m,1}$	$V_{max,2}/K_{m,2}$
dCyd	0.3	5.0	190	370	100	100
dAdo	75	300	4900	8500	9	35
dGuo	90	160	4700	7400	7	60
ara-A	1400		1400		0.1	

[a] Kinetic parameters were obtained from the results shown in Figure 4 with hexylagarose-purified T-ALL dCyd kinase. [b] Michaelis constants $K_{m,1}$ and $K_{m,2}$ and the corresponding maximum velocities were obtained by weighted linear regression of the data points in the low and high substrate concentrations. [c] The ratio V_{max}/K_m is termed efficiency, and the values with dCyd are set as 100.

Table V: Kinetic Parameters for CML dCyd Kinase[a]

substrate	K_m^{app} (μM)		V_{max}^{app} (nmol h^{-1} mg^{-1})		relative efficiency[b]	
	$K_{m,1}$	$K_{m,2}$	$V_{max,1}$	$V_{max,2}$	$V_{max,1}/K_{m,1}$	$V_{max,2}/K_{m,2}$
dCyd	0.4	5.0	6.0	8	100	100
dAdo	300	530	750	1560	16	18
dGuo	290	570	670	910	14	10
ara-A	1640		36		0.001	
ara-C	10	40	7	11	4	2

[a] DEA-cellulose-purified CML dCyd kinase was assayed with nucleosides or with ATP as variable substrates over a wide concentration range (Materials and Methods). ATP and $MgCl_2$ concentrations were 10 mM when the nucleosides were varied. When ATP concentrations were varied, $MgCl_2$ concentration was 5 mM in excess of ATP to maintain ATP–Mg^{2+} complex and dCyd and dAdo concentrations were 20 and 800 μM, respectively. The Michaelis constants $K_{m,1}$ and $K_{m,2}$, respectively, and the corresponding V_{max} values are kinetic parameters determined in the low and high substrate concentrations as described for Table IV. [b] The ratio V_{max}/K_m is termed efficiency, and the values with dCyd are set as 100.

and that this enzyme phosphorylates pyrimidine and purine deoxyribonucleosides and their analogues.

Discussion

Cytotoxic effects of purine deoxyribonucleosides and their analogues in human lymphoid cells are dependent on phosphorylation of the nucleosides (Verhoef et al., 1981; Hershfield et al., 1982). However, identity and substrate specificity of purine deoxyribonucleoside kinases in mammalian tissues have been controversial. It is clear from this study that in human T lymphoblasts and in myeloblasts there are at least three distinct purine nucleoside kinases: dCyd kinase (EC 2.7.1.74), Ado kinase (EC 2.7.1.20), and a dGuo (dAdo) kinase(s). These results are consistent with the previously noted correlation between resistance of human T lymphoblasts to both dAdo and dGuo or their arabinonucleoside analogues and defective nucleoside kinase activity (Verhoef et al., 1981; Hershfield et al., 1982). An enzyme with a high specificity for dAdo and dGuo phosphorylation has recently been reported in cultured T lymphoblasts CCRF-CEM and RPMI 8402 but not in thymus, spleen, or B lymphoblasts (Yamada et al., 1983). However, using the same T lymphoblasts CCRF-CEM, we were unable to find this T lymphoblast specific deoxynucleoside kinase by similar chromatographic procedures (Figure 1). The reason for this discrepancy is unclear. Our results are in partial agreement with studies by Hurley et al. (1983), who found that in placenta dAdo is phosphorylated by both Ado kinase and dCyd kinase. The latter enzyme appears to be the broad substrate specificity dCyd kinase (EC 2.7.1.74), since dAdo phosphorylation was inhibited by both dCyd and dGuo (Hurley et al., 1983; Table III, pool I). These investigators also reported two additional activities for dCyd and dGuo kinase in placenta that were not mutually inhibitory (Hurley et al., 1983; Table III, pool II) and that may be similar to the mitochondrial activities (peak 3) reported in this study.

dCyd kinase was found to be the major purine deoxyribonucleoside phosphorylating activity in both cultured and noncultured T lymphoblasts and in myeloblasts. The T lymphoblast dCyd kinase was purified about 2000-fold and to 10% purity and is the most purified preparation from human cells reported thus far. Data indicate that one protein, dCyd kinase, phosphorylates the pyrimidine (dCyd and *ara*-C) and the purine (dAdo, dGuo, and *ara*-A) nucleosides because (i) there is a parallel loss of dCyd, *ara*-C, dAdo, dGuo, and *ara*-A phosphorylating activities in CEM cell variants selected for resistance to *ara*-C (Verhoef et al., 1981); (ii) dCyd, dAdo, and dGuo phosphorylating activities copurify with constant ratios of dCyd to dAdo or dGuo specific activities; (iii) pyrimidine and purine deoxyribonucleoside phosphorylating activities comigrate in a single common protein band by electrophoresis; and (iv) purine and pyrimidine deoxyribonucleosides mutually inhibit phosphorylation of the other nucleoside (Sarup & Fridland, 1982).

This study is the first demonstrate that dCyd kinase shows substrate activation with both purine and pyrimidine deoxyribonucleosides. Previous investigators (Durham & Ives, 1970) may have failed to observe this because they did not use as wide a range of substrate concentrations in their kinetic studies. The substrate activation is unlikely to be due to artifacts of dCyd kinase assay, since kinetics with *ara*-A performed under the same conditions were linear with time and with protein concentration used. dCyd kinase does not appear to show substrate activation by substrate-induced oligomerization, since there was no difference in sedimentation coefficient of the enzyme when centrifuged in the absence or presence of saturating levels of magnesium–ATP and dCyd. Nonetheless, kinetic studies on dCyd kinase that is purified to homogeneity would be essential to determine whether nonlinearity in double-reciprocal plots is due to isozymes of dCyd kinase. The substrate activation kinetics of dCyd kinase suggests that this enzyme either (i) has two or more interacting catalytic sites, (ii) has a catalytic and an allosteric site, or (iii) is a hysteretic enzyme, two or more interconverting conformational states that are catalytically active and have different affinities (Neet & Ainslie, 1980).

The human lymphoblast or myeloblast dCyd kinase reported here is similar to bovine lymphoid dCyd kinase (Durham & Ives, 1970; Krenitsky et al., 1976), since the enzymes from these sources have similar molecular weights, pH optima, metal ion requirements, and substrate specificity. The inability of myloblast dCyd kinase to phosphorylate dAdo and dGuo reported (Cheng et al., 1977; Coleman et al., 1975) may be due to use of indirect assay (Cheng et al., 1977) and due to purification of dCyd kinase in the absence of the stabilizing agent DTT (Coleman et al., 1975). However, murine dCyd kinase is different from human or bovine dCyd kinases, since both genetic methods and studies with the partially purified kinase indicate that the murine enzyme does not phosphorylate dAdo and dGuo (Brockman et al., 1980; Meyers & Kreis, 1976). Substrate specificities of either lymphoid or myeloid dCyd kinase reported here show that dCyd is the best substrate, whereas purine deoxyribonucleosides dAdo and dGuo are 1 order of magnitude less efficient as substrates than dCyd. Kinetic properties of dCyd kinase indicate that in cells the enzyme is potentially capable of functioning as a highly efficient kinase for salvage of deoxynucleosides and capable of responding to a wide range of intracellular oscillations in dCyd and *ara*-C concentrations.

References

Agarwal, B. P., Sagar, S. M., & Partis, R. E. (1975) *Biochem. Pharmacol. 24*, 693–701.

Barker, J., & Lewis, R. A. (1981) *Biochim. Biophys. Acta 658*, 111–123.

Bohme, H. J., Kopperschläger, G., Schulz, J., & Hoffman, E. (1972) *J. Chromatogr. 69*, 209–214.
Brockman, R. W., Cheng, Y. C., Schabel, F. M., Jr., & Montgomery, J. A. (1980) *Cancer Res. 40*, 3610–3615.
Carson, D. A., Wasson, D. B., Lakow, E., & Kamani, N. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 3849–3852.
Chang, C. H., Brockman, R. W., & Bennett, L. L., Jr. (1980) *J. Biol. Chem. 255*, 2366–2371.
Cheng, Y. C., Domin, G., & Lee, L. S. (1977) *Biochim. Biophys. Acta 481*, 481–492.
Coleman, N. C., Stoller, R. G., Drake, J. C., & Chabner, B. A. (1975) *Blood 46*, 791–803.
Cornish-Bowden, A. (1979) *Fundamentals of Enzyme Kinetics*, pp 51–52, Butterworths, London.
Durham, J. P., & Ives, D. H. (1970) *J. Biol. Chem. 245*, 2276–2284.
Gabriel, O. (1971) *Methods Enzymol. 22*, 565–578.
Giblett, E. R., Anderson, T. E., Cohen, F., Pollara, B., & Meuwissen, H. J. (1972) *Lancet 2*, 1067–1069.
Giblett, E. R., Ammann, A. J., Sandman, R., Wara, D. W., & Diamond, L. K. (1975) *Lancet 1*, 1010–1013.
Gower, W. R., Jr., Carr, M. C., & Ives, D. H. (1979) *J. Biol. Chem. 254*, 2180–2183.
Hershfield, M. S., Fetter, J. E., Small, W. C., Bagnara, A. S., Williams, S. R., Ullman, B., Martin, D. W., Jr., Wasson, D. B., & Carson, D. A. (1982) *J. Biol. Chem. 257*, 6380–6386.
Hurley, M. C., Paletta, T. D., & Fox, I. H. (1983) *J. Biol. Chem. 258*, 15021–15027.
Ives, D. H., Durham, J. P., & Tucker, V. S. (1969) *Anal. Biochem. 28*, 192–205.
Johnson, D., & Lardy, H. (1967) *Methods Enzymol. 10*, 94–96.
Kim, B. Y., Chu, S., & Partis, R. E., Jr. (1968) *J. Biol. Chem. 243*, 1763–1770.
Krenitsky, T. A., Tuttle, J. V., Koszalka, G. W., Chen, I. S., Reachman, L. M., Rideout, J. L., & Elion, G. R. (1976) *J. Biol. Chem. 251*, 4055–4061.
Lindberg, B., Klenow, H., & Hansen, K. (1967) *J. Biol. Chem. 242*, 350–356.
Martin, R. G., & Ames, B. N. (1961) *J. Biol. Chem. 226*, 1372–1379.
Meyers, M. B., & Kreis, W. (1976) *Arch. Biochem. Biophys. 177*, 10–15.
Miller, R. L., Adanczyk, D. L., Miller, W. H., Koszalka, G. W., Rideout, T. L., Reachman, L. M., Chao, F. Y., Haggerty, J. J., Krenitsky, T. A., & Elion, G. R. (1979) *J. Biol. Chem. 254*, 2346–2352.
Neet, K. E., & Ainslie, R. G., Jr. (1980) *Methods Enzymol. 64*, 192–226.
Sarup, J., & Fridland, A. (1982) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 41*, 1397.
Schnehli, H. P., Hill, D. L., & Bennett, L. L., Jr. (1967) *J. Biol. Chem. 242*, 1997–1004.
Srere, P. A. (1969) *Methods Enzymol. 13*, 3–11.
Ullman, B., Levinson, B. B., Hershfield, M. S., & Martin, D. W., Jr. (1981) *J. Biol. Chem. 256*, 848–852.
Vallee, B. L., & Hoch, F. L. (1955) *Proc. Natl. Acad. Sci. U.S.A. 52*, 327–330.
Verhoef, V., Sarup, J. C., & Fridland, A. (1981) *Cancer Res. 41*, 4478–4486.
Yamada, Y., Goto, H., & Ogarawara, N. (1980) *Biochim. Biophys. Acta 616*, 199–207.
Yamada, Y., Goto, H., & Ogasawara, N. (1983) *Biochim. Biophys. Acta 761*, 34–40.

Stereochemistry and Mechanism of a New Single-Turnover, Half-Transamination Reaction Catalyzed by the Tryptophan Synthase $\alpha_2\beta_2$ Complex

Edith Wilson Miles

Laboratory of Biochemical Pharmacology, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland 20892

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ABSTRACT: Tryptophan synthase is a versatile enzyme that catalyzes a wide variety of pyridoxal phosphate dependent reactions that are also catalyzed in model systems. These include β-replacement, β-elimination, racemization, and transamination reactions. We now show that the apo-$\alpha_2\beta_2$ complex of tryptophan synthase will bind two unnatural substrates, pyridoxamine phosphate and indole-3-pyruvic acid, and will convert them by a single-turnover, half-transamination reaction to pyridoxal phosphate and L-tryptophan, the natural coenzyme and a natural product, respectively. This enzyme-catalyzed reaction is more rapid and more stereospecific than an analogous model reaction. The *pro-S* 4′-methylene proton of pyridoxamine phosphate is removed during the reaction, and the product is primarily L-tryptophan. We conclude that pyridoxal phosphate enzymes may be able to catalyze some unnatural reactions involving bound reactants and bound coenzyme since the coenzyme itself has the intrinsic ability to promote a variety of reactions.

We are examining the reaction specificity of the tryptophan synthase $\alpha_2\beta_2$ complex of *Escherichia coli* (EC 4.1.2.20) and the stereochemistry of the reactions catalyzed in order to understand the relative roles of the protein and of the pyridoxal phosphate coenzyme. This study was designed to test whether the apo-$\alpha_2\beta_2$ complex formed by removal of pyridoxal phosphate from the holo-$\alpha_2\beta_2$ complex will catalyze the single-turnover transamination reaction 1. Although pyridoxamine phosphate and indole-3-pyruvic acid are not natural substrates of this enzyme, the products pyridoxal phosphate and L-