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ALTERED PROPERTIES OF HUMAN T-LYMPHOBLAST SOLUBLE LOW K_m 5′-NUCLEOTIDASE: COMPARISON WITH B-LYMPHOBLAST ENZYME

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Abstract—Soluble low K_m 5′-nucleotidases have been purified from human cultured T- and B-lymphoblasts to compare their properties and to examine the mechanism of different rates of nucleotide dephosphorylation. The enzyme from B-lymphoblasts (MGL-8) was 4385-fold purified with a specific activity of 114 μmol/min/mg, while the enzyme from T-lymphoblasts (CEM, MOLT-4) was 4355-fold purified with a specific activity of 35 μmol/min/mg. The activity of both enzymes have an absolute requirement for Mg^{++}. The B-cell enzyme has maximum activity with $Mg^{2+} > Mn^{2+} > Co^{2+}$, while the T-cell enzyme had maximum activity with $Co^{2+} > Mn^{2+} > Mg^{2+}$. The optimum activity was at pH 7.4–9.0 for the B-cell enzyme and pH 9.0 for the T-cell enzyme. Substrate specificity was the same for both enzymes with the following relative V_{max} values:

CMP > UMP > dUMP > dCMP > dAMP > IMP > GMP > dIMP > dGMP.

The K_m values for AMP and IMP were 12 and 25 μM for the B-cell enzyme, and 7.0 and 12 μM for the T-cell enzyme. ATP and ADP are competitive inhibitors of these enzymes with apparent K_i values of 100 and 20 μM for the B-cell enzyme, and 44 μM and 8 μM for the T-cell enzyme, respectively. The apparent molecular mass by gel filtration column chromatography is 145 kD for the B-cell enzyme and 72 kDa for the T-cell enzyme. The subunit molecular masses by Western blots are 69.2 kD for both enzymes.

These properties suggest that the B-lymphoblast enzyme is identical or similar to the enzyme from human placenta. However, the T-cell enzyme has some different properties. We conclude that these differences plus a lower content of low K_m 5′-nucleotidase in T-cells may account for the decreased ability of T-lymphoblasts to dephosphorylate nucleotides and may contribute to the selective cytotoxicity of deoxyribonucleosides for T-lymphoblasts as compared to B-lymphoblasts.

Key words: 5′-nucleotidase, nucleotide degradation, nucleoside toxicity, leukemic cells.

INTRODUCTION

INTRACELLULAR nucleotide degradation in human cells is highly regulated [1–3]. A key step in this pathway is dephosphorylation of nucleotide 5′-monophosphates catalyzed by 5′-nucleotidase (5′-ribonucleotide phosphohydrolase, EC 3.1.3.5). This enzyme is important for intracellular nucleotide metabolism and adenosine release [4–6].

Available data indicate that 5′-nucleotidase from human T-lymphoblastoid cell lines has a low activity as compared to B-lymphoblastoid cell lines [7]. In T-lymphoblasts, intracellular nucleoside 5′-monophosphate dephosphorylation is lower compared with B-lymphoblasts or with T- and B-lymphocytes [5, 8–10]. Consequently, under stress conditions, B-cells selectively release adenosine [11]. In addition, the activity of 5′-nucleotidase may have prognostic value in leukemias [12–15] and other diseases: it is decreased in the lymphocytes of immunodeficient patients with hypogammaglobulinemia [16–18] and patients with chronic lymphocytic leukemia [19], and in lymphoblasts of patients with acute T-cell lymphoblastic leukemia [12, 20]. Low intracellular 5′-nucleotidase activity may contribute to the accumulation of toxic deoxyribonucleoside triphosphates in immunodeficient patients with adenosine deaminase defi-

Abbreviations: BCIP, 5-bromo-4-chloro-3-indolyl phosphate; *NBT*, nitro blue tetrazolium; *MBS*, *m*-maleimidobenzoyl-*N*-hydroxysuccinate ester; *E-64*, *N*-[*N*-(*L*-3-trans-carboxyoxiran-2-carbonyl)-L-leucyl]-agmatine; *EGTA*, ethyleneglycol-bis-(β-aminoethyl ether) *N*,*N*,*N*¹,*N*¹-tetraacetic acid; *PMSF*, phenylmethylsulfonylflouride; *SDS-PAGE*, sodium dodecyl phosphate polyacrylamide gel electrophoresis.

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ciency, or in the presence of adenosine deaminase inhibitors and with purine nucleoside phosphorylase deficiency [21–23].

The basis for the decreased dephosphorylating activity in human T-lymphoblasts is unclear. The 5′-phosphomonoesterase activity is a composite of nonspecific phosphatases and specific 5′-nucleotidases. There are two soluble specific 5′-nucleotidases in lymphoid tissue: low K_m and high K_m forms [24]. Our previous studies determined that the decrease in 5′-nucleotidase in T-lymphoblasts is apparently related to the low K_m form [24]. Therefore, as one approach to examine the decreased nucleoside 5′-monophosphate activity of these cells, we have tested the hypothesis that there is an alteration of specific soluble low K_m 5′-nucleotidase in T-lymphoblasts as compared to B-lymphoblasts. To accomplish this, we have adapted our purification and characterization of human placental and rat liver low K_m 5′-nucleotidase [25, 26] to cultured B-lymphoblast and T-lymphoblast soluble 5′-nucleotidases.

EXPERIMENTAL PROCEDURE

Materials

[8-^{14}C]AMP (50 mCi/mmol) and [8-^{14}C]IMP (50 mCi/mmol) were purchased from Amersham Corporation (Chicago, IL). All nucleosides, 5′-monophosphates, ADP, ATP, adenosine, inosine, p-nitrophenylphosphate, gel filtration protein standards, imidazole, β-mercaptoethanol, trizma base, α-methyl-D-mannoside, bovine serum albumin, goat anti-rabbit affinity purified IgG conjugated with alkaline phosphatase trypsin inhibitor (soybean), 1,10-phenanthroline, α,β-methylene adenosine diphosphate (AMPCP), α,β-methylene adenosine triphosphate (AMPCPP), β,γ-methylene adenosine triphosphate (AMPPCP), 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitro blue tetrazolium (NBT) were purchased from Sigma Chemical Co. (St Louis, MO). AMP-Sepharose 4B, Concanavalin A (Con A)-Sepharose., Sephacryl S-300, Sephadex G-25, blue dextran and columns were obtained from Pharmacia Fine Chemicals (Piscataway, NJ). Bradford protein reagent, acrylamide, *N,N′*-methylene bisacrylamide, *N,N,N′,N′*-tetramethylethylenediamine, ammonium persulfate, bromophenol blue, SDS-polyacrylamide gel protein standard kits of high and low molecular weight, biotinylated molecular weight standards and streptavidin conjugated with alkaline phosphatase, and the silver stain reagent kit were purchased from Bio Rad (Rockville Center, NY). The pressure ultrafiltration unit with YM-10 membranes was obtained from Amicon (Danvers, MA). All other reagents used were of the highest quality generally available.

Cell lines and cell culture

Three human lymphoblastoid cell lines were used for our studies including T-lymphoblasts (CEM and MOLT-4) and B-lymphoblasts (MGL-8). The MGL-8 (B-lymphoblasts) cell line was derived from a normal individual and was a gift from the laboratory of Dr John Littlefield, Johns Hopkins University (Baltimore, MD). MOLT-4 and CEM (T-lymphoblasts) cell lines were obtained from HEM Research, Inc., Rockville, MD, and were originally derived from patients with acute lymphoblastic leukemia. All cell lines were grown in suspension culture using RPMI-1640 media supplemented with 10% donor calf serum and 2 mM L-glutamine. The cells were maintained in logarithmic growth phase at 37°C and 5% CO_2. Mycoplasma contamination was excluded by periodic microbiology tests.

Enzyme assays

Protein containing extracts of soluble 5′-nucleotidase were incubated for 30 min or 60 min at 37°C in a reaction mixture containing 62.5 mM imidazole-HCl pH 7.4, 20 mM $MgCl_2$, 1 mg/ml bovine serum albumin, and 0.2 mM [8-^{14}C]AMP or [8-^{14}C]IMP in a total volume of 100 μl. After stopping the reaction by heating at 85°C for 2 min, precipitated protein was removed by centrifugation. Twenty microliters were spotted on Whatman 3 MM chromatography paper with 20 μl nonradioactive nucleosides and nucleotides (1 mg/ml). Nucleosides and nucleotides were separated by high voltage electrophoresis at 4000 V and 250 mA for 30 min in 50 mM sodium borate pH 9. Nucleoside and nucleotide spots were located with an ultraviolet light, cut out and counted in LKB Rackbeta liquid scintillation spectrometer. In addition, enzyme activity was measured by release of inorganic phosphate (Pi) in incubation media of 200 μl containing 67 mM imidazole-HCl pH 7.4, 20 mM $MgCl_2$, 1 mg/ml bovine serum albumin and 5 mM AMP. The reaction was stopped by the addition of 50 μl of 10% trichloroacetic acid. The amount of Pi liberated was determined by the method of Chen *et al.* [27].

Non-specific phosphatase activity was measured in an incubation mixture containing 62.5 mM imidazole pH 7.4, 20 mM $MgCl_2$, 1 mg/ml bovine serum albumin and 12.5 mM p-nitrophenylphosphate. The reaction was incubated for 30 min at 37°C and stopped with the addition of 0.8 ml 0.4 M NaOH. The samples were clarified by centrifugation and the optical density measured at 400 nm on a Gilford 260 spectrophotometer.

Protein assay

Protein concentration was estimated by the method of Bradford [28] with alcohol dehydrogenase as the standard. Proteins eluting from the columns during the various purification steps were monitored by absorbance at 280 nm or by the Bradford method.

Preparation of cell extracts

Cells were collected by centrifugation, washed three times, and resuspended in 0.25 M sucrose, 1 mM $MgCl_2$, and 10 mM Tris-HCl pH 7.4. The cell pellet was stored at −70°C. The frozen cells were thawed, suspended in 20 mM imidazole-HCl, pH 7.0, 20 mM $MgCl_2$ (buffer A) to 5×10^8 cells/ml, and were lysed by nitrogen bomb cavitation (1000 lb./in.^{2} for 30 min at 4°C) and by Dounce tissue homogenizer (about 30–40 strokes). The lysate was spun for 10 min at 10,000 × *g* to remove cellular debris. The supernatant was spun at 50,000 × *g* for 2 h to eliminate the remaining cellular membranes and then was dialyzed overnight at 4°C against 10 mM Tris-HCl pH 7.4, and 20 mM $MgCl_2$. This lymphoblast supernatant was then used immediately for enzyme purification.

Enzyme purification

The purification used was similar to our procedure for human placental soluble 5′-nucleotidase [25]. The lymphoblast supernatant (50 ml) was passed through an AMP-Sepharose 4B affinity chromatography column. Soluble 5′-nucleotidase activity was eluted from the column with 60 ml of 10 mM ADP in 20 mM imidazole-HCl pH 7.0 and 20 mM $MgCl_2$ (buffer A). The eluate was concentrated from 25 ml to 2 ml using an AMICON pressure ultrafiltration unit with YM-10 membrane.

Concentrate (2 ml) from the AMP-Sepharose column was then applied to a Sephacryl S-300 gel filtration column equilibrated with buffer A. The fractions with the highest activity were pooled (17 ml) and applied to concanavalin A-Sepharose 4B column. This column was then washed with 45 ml of 20 mM imidazole-HCl pH 7.0 and 20 mM $MgCl_2$. The enzyme was eluted in a single peak with 45 ml of 0.5 M α-methylmannoside at 37°C in buffer A. The eluate from the concanavalin A-Sepharose was immediately concentrated to 1.5 ml to preserve enzyme stability. This preparation was stored at 4°C and used for our characterization studies.

Modifications of the purification were made to obtain proteins for subunit molecular weight determinations by Western blot. The T- and B-lymphoblast extract was applied to 20 ml concanavalin A-Sepharose columns according to the procedure described for rat liver 5′-nucleotidase purification [26]. The pooled, concentrated and dialyzed active fractions were passed through a DE-52 ion exchange column. The preparations after DE-52 chromatography were taken for Western blot analysis.

Molecular weight determination

The molecular weight of the native purified proteins were determined by gel filtration chromatography on a Sephacryl S-300 column (1.6 × 70 cm) equilibrated with 5% glycerol, 20 mM $MgCl_2$, 60 mM imidazole-HCl pH 7.0, and 0.02% sodium azide. Two milliliter samples were applied, the column flow rate was 6 ml/h, and 1.75 ml fractions were collected. We used blue dextran and dinitrophenylalanine to determine the void volume and exclusion volume, respectively. The void volume was 55 ml and the excluded volume was 150 ml. The column was standardized with aldolase (Mr = 140,000), bovine serum albumin (Mr = 67,000), ovalbumin (Mr = 43,000), alpha-chymotrypsinogen A (Mr = 25,000), and cytochrome C (Mr = 12,000). The protein was estimated by absorbance at 280 nm. K_{av} was calculated for each standard according to the method of Laurent and Kilander [29] and Siegel and Monty [30]. The molecular weights and Stokes radius were determined from the plot of K_{av} vs the molecular weight and Stokes radius of the standard proteins.

Subunit molecular weight determination

Western blot procedure was used to determine the molecular weights of T- and B-lymphoblast 5′-nucleotidase.

Rabbit antibodies raised against a peptide derived from human placental soluble low K_m 5′-nucleotidase were used to bind to the enzyme protein immobilized on nitrocellulose. A peptide for amino acid sequence analysis was prepared by trypsin digestion of pure 5′-nucleotidase purified to 800 μmol/min/mg protein specific activity. The sequence analysis yielded a 17 amino acid sequence. The synthetic peptide with attached cysteine at the *N*-terminus was conjugated with keyhole limpet hemocyanin (KLH, 50:50 w/w). One milligram of conjugate with complete Freund's adjuvant (1 ml of total volume) was injected subcutaneously to the rabbit at 13 different spots. One-half milligram of peptide-KLH conjugate and incomplete Freund's adjuvant were injected every 4 weeks and the blood was drawn 10 days after each injection.

Polyacrylamide slab gel electrophoresis with human placenta, B-and T-lymphoblast soluble low K_m 5′-nucleotidase and biotynylated standards was run with sodium dodecylsulfate according to standard procedure [31] and the gel was electroblotted with Bio-Rad Trans-blot cell at 60 V for 6 h. The nitrocellulose was blocked with 5% dry milk overnight and incubated with 1:1000 rabbit serum for 3 h. The bands were developed with goat anti-rabbit alkaline phosphatase conjugated antibodies. Biotinylated standards with avidin conjugated with alkaline phosphatase were used to determine molecular weight. Color reaction was developed with BCIP and NBT as described [32]. The molecular weight of the enzymes was determined by measuring R_f values and comparing them to the R_f values of known biotinylated protein standards (Bio-Rad): phosphorylase B (97.4 kD), bovine serum albumin (66.2 kD), ovalbumin (42.7 kD), carbonic anhydrase (31.0 kD) soybean trypsin inhibitor (21.5 kD), and lysozyme (14.4 kD).

Kinetics and calculations

The K_m values were determined from initial velocity studies. For each study there were at least six different substrate concentrations. Each determination was carried out 2–4 times in duplicate. Double reciprocal plots of initial velocity vs the substrate concentration were used to estimate the K_m values. Linearity with time was established

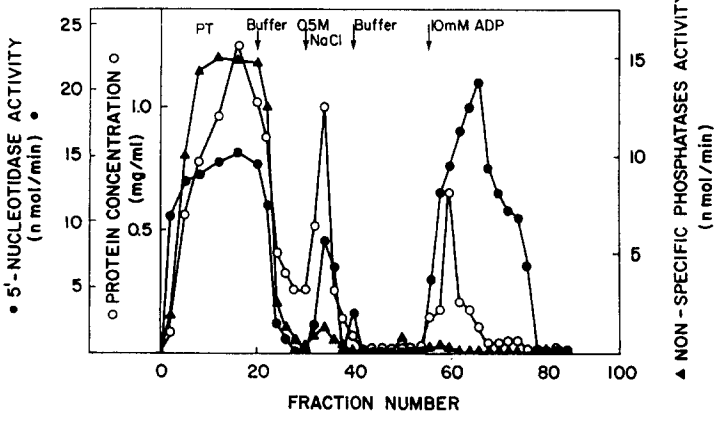

FIG. 1. 5′-AMP-Sepharose 4B affinity chromatography. Forty milliliters of either T- or B-lymphoblast supernatants (shown for B-lymphoblasts only) were applied to 20 ml 5′-AMP-Sepharose 4B affinity columns (2.5 × 7 cm). The column was equilibrated with buffer A (see Experimental Procedure). After application of the enzyme extract, the column was washed with 120 ml buffer A to remove unbound proteins and then was washed with 60 ml 0.5 M NaCl in buffer A to remove non-specifically bound proteins. Subsequently, the bound soluble 5′-nucleotidase was eluted with about 90 ml of 10 mM ADP in buffer A. The active fractions were pooled, concentrated using an Amicon YM-10 filter.

TABLE 1. PURIFICATION OF SOLUBLE LOW K_m 5'-NUCLEOTIDASE FROM B-LYMPHOBLASTS (MGL-8)

Step	Total protein (mg)	Specific activity (μmol/min/mg)	Total enzyme (μmol/min)	Purification (fold)	Recovery (%)
Lymphoblast supernatant	351	0.026	8.3	—	100
AMP Sepharose-4B	7.62	0.840	6.4	32	76
AMP-Sepharose concentrate	5.18	0.820	4.3	32	51
Sephacryl S-300	0.15	33.36	4.2	1283	50
Con A-Sepharose concentrate	0.04	114.0	4.0	4385	48

TABLE 2. PURIFICATION OF SOLUBLE LOW K_m 5'-NUCLEOTIDASE FROM T-LYMPHOBLASTS (MOLT-4)

Step	Total protein (mg)	Specific activity (μmol/min/mg)	Total enzyme (μmol/min)	Purification (fold)	Recovery (%)
Lymphoblasts supernatant	637	0.008	5.1	—	100
AMP Sepharose-4B	20.5	0.212	4.4	27	87
AMP-Sepharose concentrate	20.0	0.220	4.3	30	83
Sephacryl S-300	0.10	17.4	1.8	2176	36
Con A-Sepharose concentrate	0.05	34.7	1.7	4335	34

for different concentrations used in initial velocity studies for K_m determinations. Lineweaver–Burk plots of initial velocity vs concentration of substrate were linear. Kinetic data were fitted to the simple Michaelis–Menten equation, i.e. a hyperbola, by a modification of Cleland's program (Cleland, 1967), on a Vax 11/730 minicomputer.

RESULTS

Purification

Both enzymes showed a similar purification profile (Fig. 1). The initial step of purification with AMP-Sepharose affinity chromatography removes all non-specific phosphatase activity from the crude preparation, since these enzymes pass through the column. This step shows consistent elution of a single peak of 5'-nucleotidase activity. The enzyme activity peak elutes from the gel filtration column in a same major protein peak (data not shown) separated from other proteins.

The purification of soluble 5'-nucleotidase from B-lymphoblasts yielded a 4385-fold purified enzyme with a specific activity of 114 μmoles/min/mg protein (Table 1). The final recovery was 48%. Gel filtration column chromatography yielded a 40-fold purification with a recovery of 68%. Con-A-Sepharose affinity chromatography yielded a 3.5-fold purification with a consistent 96% recovery.

The purification of cytoplasmic 5'-nucleotidase from T-lymphoblasts (MOLT-4) yielded 4335-fold purified enzyme with a specific activity of 40 μmol/min/mg, and final recovery of 34% (Table 2). In this case the gel filtration column also yielded a 72-fold purification, with a 43% recovery. Concanavalin A-Sepharose chromatography yielded a 2.0-fold purification with a 90% recovery. The enzyme purified from CEM T-lymphoblasts had identical properties.

TABLE 3. EFFECT OF DIVALENT CATIONS ON CYTOPLASMIC LOW K_m 5'-NUCLEOTIDASE ACTIVITY FROM T- AND B-LYMPHOBLASTS

Divalent cation (7.5 mM)	Enzyme activity* (% of control)		
	B-lymphoblasts (MGL-8)	T-lymphoblasts (CEM)	T-lymphoblasts (MOLT-4)
Mg	100	100	100
Mn	79	107	115
Co	78	142	132
Ba	74	23	28
Ca	72	12	7
Cu	57	60	57
Ni	55	53	47
Zn	34	56	53
Fe	33	15	14

* The activity is expressed as the percent of the value with Mg which have the following in μmol/min/mg: MGL-8, 110; CEM, 37: MOLT-4, 35.

Divalent cations

The enzyme was dialyzed against 10 mM Tris-HCl pH 7.4 and 0.2 mM EDTA and was assayed with different divalent cations at 7.5 mM (Table 3). There was no activity of these enzymes without Mg^{++}. As compared to Mg^{++}, the B-lymphoblast enzyme was 79% effective with Mn and was 78% effective with

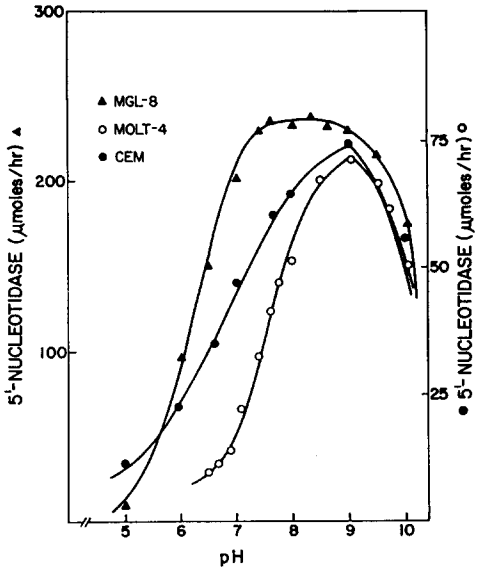

FIG. 2. pH activity profile of T- and B-lymphoblasts soluble low K_m 5′-nucleotidases. The enzymes were assayed under standard conditions at different pH values using 100 μM AMP as substrate. The B-lymphoblast enzyme has an optimum pH plateau from pH 7.4 to 9.0, while both T-lymphoblast enzymes have a pH optimum of 9.0.

Co^{++}. In contrast, the T-cell enzyme showed a greater preference for cobalt (132–142%) and manganese (107–115%). The B-lymphoblast enzyme had substantially more activity than the T-lymphoblast enzyme with the addition of Ba, Ca or Fe.

Both enzymes displayed an activation curve with magnesium, reaching its maximum activity between 10 and 20 mM $MgCl_2$-. No AMP dephosphorylation occurred without magnesium.

pH optimum

Soluble 5′-nucleotidase purified from B-lymphoblasts had maximum activity at pH 7.4 and remained at a plateau up to pH 9.0 (Fig. 2). In contrast, the T-lymphoblast enzyme with the same experimental conditions has its maximum activity peak at pH 9.0.

TABLE 4. ACTIVITY OF CYTOPLASMIC LOW K_m 5′-NUCLEOTIDASE FROM T- AND B-LYMPHOBLASTS WITH VARIOUS SUBSTRATES

Substrate (2.5 mM)	Enzyme activity (% of control) B-lymphoblasts (MGL-8) (μmol/min/mg)	T-lymphoblasts (CEM) (μmol/min/mg)	T-lymphoblasts (MOLT-4) (μmol/min/mg)
CMP	240.8 (247)*	25.3 (248)	14.7 (233)
UMP	237.4 (244)	24.1 (236)	13.3 (211)
dUMP	209.0 (215)	13.7 (135)	7.3 (116)
dCMP	203.0 (208)	16.3 (160)	7.8 (124)
dAMP	101.0 (104)	12.3 (119)	6.4 (102)
AMP	97.4 (100)	10.2 (100)	6.3 (100)
IMP	95.0 (98)	8.9 (87)	4.4 (70)
GMP	91.0 (93)	8.3 (81)	4.2 (67)
dIMP	66.0 (68)	8.0 (78)	3.1 (49)
dGMP	46.0 (47)	6.6 (65)	6.9 (110)
TMP	—	4.9 (48)	3.0 (48)

* The values in brackets are percentages calculated using the activity with AMP as 100%.

Substrate specificity

When soluble 5′-nucleotidases from T- and B-lymphoblasts were assayed with different nucleoside 5′-monophosphates at the same concentration of 2.5 mM, the enzymes showed high activity with CMP, UMP, dCMP and dUMP (Table 4). There is no difference in the substrate preference.

Kinetic properties

Initial velocity studies were performed with dif-

TABLE 5. KINETIC VARIABLES FOR CYTOPLASMIC LOW K_m 5′-NUCLEOTIDASES FROM T- AND B-LYMPHOBLASTS

Compound	B-lymphoblast enzyme (MGL-8)		T-lymphoblast enzyme (CEM)	
	K_m (μM)	K_i app (μM)	K_m (μM)	K_i app (μM)
AMP	12	—	7.0	—
IMP	25	—	10	—
ADP	—	20	—	8.0
ATP	—	110	—	55
Pi	—	23,000	—	16,000
	% inhibition			
AMPCP (100 μM)	70		73	
AMPCPP (100 μM)	10		25	
AMPPCP (100 μM)	10		12	

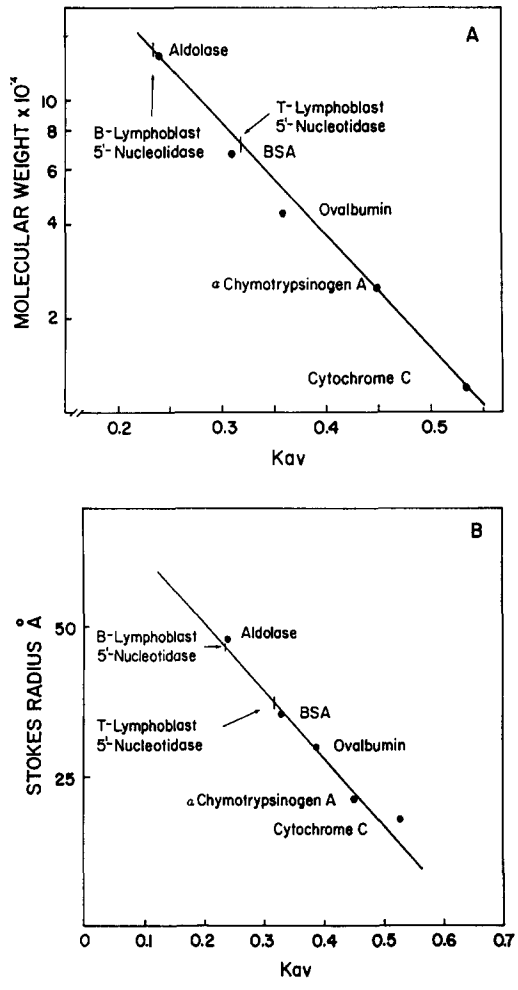

FIG. 3. Molecular weight and Stokes radii of T- and B-lymphoblast soluble low K_m 5'-nucleotidases on Sephacryl S-300. A Sephacryl S-300 gel filtration column was calibrated as described in Experimental Procedure, using cytochrome C (12.4 kD), alpha-chymotrypsinogen (25 kD), ovalbumin (43 kD), bovine serum albumin (67 kD), and aldolase (140 kD). The apparent molecular mass of the B-lymphoblast enzyme is 145 kD as compared to the T-lymphoblast enzyme of 72.5 kD (panel A). The Stokes radius of the B-lymphoblast enzyme is 47.4 A, while the T-lymphoblast enzyme is 37.0 (panel B).

ferent concentrations of AMP or IMP. Soluble 5'-nucleotidases purified from T- and B-lymphoblasts displayed hyperbolic kinetics. Double reciprocal plots of initial velocity studies are linear. The K_m values for the B-lymphoblast enzyme are 12 μM for AMP and 25 μM for IMP. The T-lymphoblast enzyme showed K_m values of 7 μM and 12 μM for AMP and IMP, respectively (Table 5).

Inhibition studies of soluble 5'-nucleotidases were performed at different concentrations of AMP and fixed concentrations of ATP (100 μM), ADP (25 μM) and Pi (25 mM). With double reciprocal plots of initial velocity data, ATP or ADP produce changes in the slope only consistent with competitive inhibition. The apparent K_i values were 100 and 20 μM for ATP and ADP, respectively, for the B-lymphoblast enzyme. The apparent K_i values were 55 μM for ATP and 8 μM for ADP for the T-lymphoblast enzyme. Inhibition studies with Pi show changes in both the slope and intercept, suggesting non-competitive inhibition. The apparent K_i values were 23 mM (slope), and 44 mM (intercept) for the B-cell enzyme, and 16 mM (slope) and 38 mM (intercept) for the T-cell enzyme (Table 5).

Molecular weight determination

The native molecular weights of T- and B-lymphoblast cytoplasmic 5'-nucleotidases were estimated from the elution of the known molecular weight standards from the Sephacryl S-300 gel filtration column. The elution volume of B-lymphoblast soluble 5'-nucleotidase indicates a molecular mass of 145 kD with a Stokes radius of 47.4 Å (Figs 3 and 4). The enzyme from T-lymphoblasts was found to have a molecular mass of 72.5 kD with a Stokes radius of 37.0 Å (Figs 3 and 4).

Subunit molecular weight

The Western blot analysis yielded a molecular mass of 69.2 kD for B- and T-lymphoblast 5'-nucleotidases. This compares with 70.6 kD by the same procedure for human placental enzyme (Fig. 5).

DISCUSSION

Cultured human T- and B-lymphoblasts provide a model system for critical examination of the relationships between nucleotide degradation and 5'-nucleotidase activities. T-lymphoblasts have a decreased ability to dephosphorylate intracellular nucleotides as compared to B-lymphoblasts [5, 8, 10]. In addition, T-lymphoblasts have a lower activity of soluble and membrane bound 5'-nucleotidases (low K_m type) relative to B-lymphoblasts [5, 7–10, 24, 34]. The discovery of leukemias and immunodeficiency states with absent or low levels of 5'-nucleotidase activity has created further interest in its metabolic significance [12, 16–21, 23].

Our observations have elucidated a number of distinguishing properties between soluble low K_m 5'-nucleotidase from T- and B-lymphoblasts on the one hand and between lymphoblast and human placental enzyme on the other.

Firstly, there is a substantial difference in the AMP-dephosphorylating activity between these cells. The B-cell enzyme is several times more active than the T-cell enzyme in crude extracts as previously described [5, 10, 20]. Furthermore, the pH activity

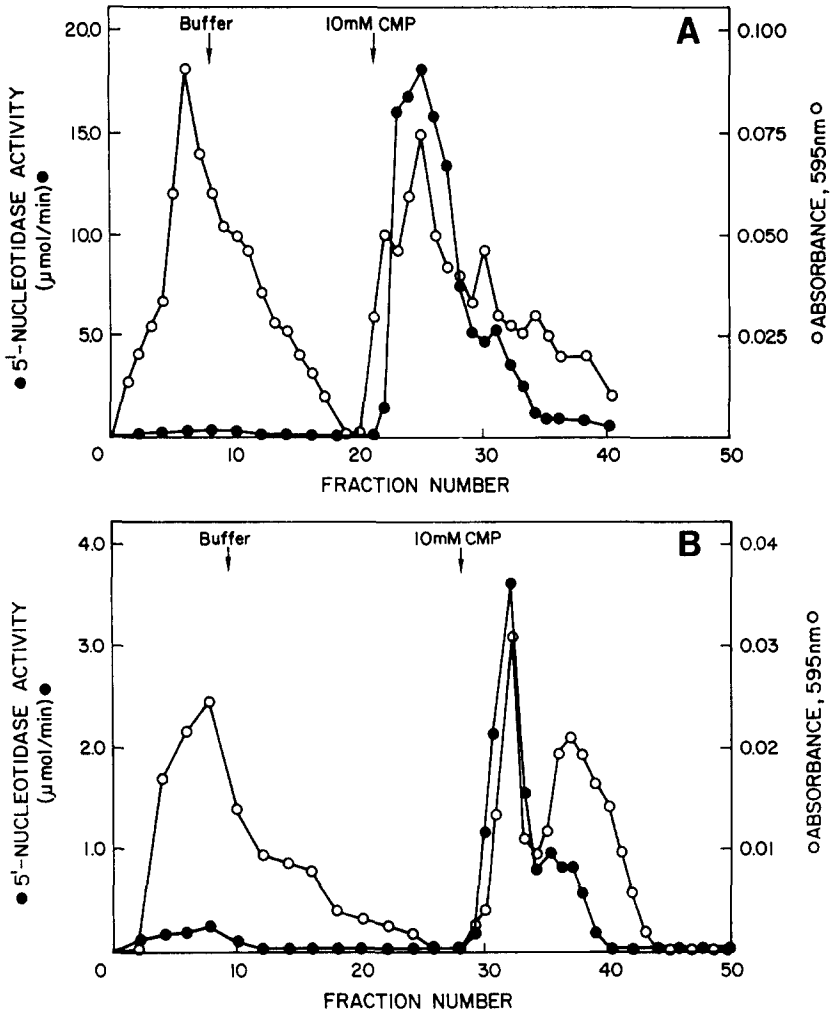

FIG. 4. 5′-AMP-Sepharose 4B affinity chromatography. Eluates from concanavalin A-Sepharose were applied to a second AMP-Sepharose 4B chromatography column. The columns were washed with 30 ml of buffer A and the enzymes were eluted with 15 ml of 10 mM CMP in buffer A. Panel A shows the profile for B-cell enzyme (MGL-8), while panel B the profile for T-cell enzyme (MOLT-4).

profiles are different for soluble 5′-nucleotidase isolated from T- and B-lymphoblasts. The T-lymphoblast enzyme pH activity peak most closely resembles alkaline phosphatase [35]. In contrast, the B-lymphoblast 5′-nucleotidase is similar to the soluble 5′-nucleotidase from human placenta and bovine liver which have a plateau of activity from pH 7.4 to 9.0 [36]. This is different from the crude soluble preparations of T- and B-lymphoblasts and the high K_m enzyme partially purified from lymphoblasts, human malignant lymphocytes, and rat and chicken liver, which have pH optima from 6.3 to 6.5 [5, 8, 9, 37–40].

Several catalytic properties of the B-lymphoblast enzyme are similar to the human soluble placental 5′-nucleotidase [25]. They have similar K_m values for AMP and IMP, similar K_i values for ATP, ADP and inorganic phosphate. Our results contrast with previously observed K_m values of 330 μM for AMP with crude soluble preparation of B-cells, and K_m values of 620 μM or higher for AMP with partially purified from lymphoid cells [8, 9]. The latter observation relates to the high K_m 5′-nucleotidases. Cytoplasmic high K_m 5′-nucleotidases from other sources have K_m values for AMP and IMP, which are one or two orders of magnitude higher [8, 9, 38–42].

Although both low K_m 5′-nucleotidases have an absolute requirement for magnesium, divalent cation preference distinguishes T- and B-lymphoblast low K_m 5′-nucleotidases. B-lymphoblast 5′-nucleotidase

FIG. 5. Western blot of soluble low K_m 5′-nucleotidase from human B-lymphoblasts (A), T-lymphoblasts (B) and placenta (C). A 7.5% gel was electroblotted onto nitrocellulose, reacted with rabbit anti-peptide monospecific antibodies and visualized with alkaline phosphatase-conjugated goat anti-rabbit IgG and BCIP and NBT staining [32]. Pure soluble low K_m 5′-nucleotidase from human placenta was included for comparison.

has the highest activity with magnesium as has been observed with other soluble 5′-nucleotidases and membrane 5′-nucleotidases [9, 25, 37–40]. In contrast, the T-cell enzyme has maximum activity with cobalt and manganese. In addition, the enzymes are different by virtue of their preference for Ca, Ba or Fe.

The substantial differences in catalytic properties of T- and B-lymphoblast enzymes was supported by a major alteration in molecular weight. Soluble 5′-nucleotidase isolated from B-cells is similar to a number of mammalian enzymes with low K_m 5′-nucleotidase activity [25, 26, 44]. The native protein is a dimer of 145 kD of molecular mass and 69.2 kD for each subunit. On the other hand, the enzyme from T-cells appears to be a monomer with molecular mass of 69.2–72.5 kD. Structural differences related to carbohydrate moiety have been already shown for structurally related plasma membrane 5′-nucleotidase [45]. Whether this is the case for these two enzymes and whether the altered catalytic properties of the enzyme from T-cells are related to the inability to form a dimeric structure remains to be established. In an attempt to investigate if there is another and/or different species of mRNA coding for low K_m 5′-nucleotidase in T-cells we have performed a Northern blot analysis. We have been unable to detect any level of mRNA for low K_m 5′-nucleotidase in B- and T-cells with up to 30 μg total RNA and human placenta low K_m cDNA as a probe (data not shown).

There are some important biological implications of the reduced and altered low K_m 5′-nucleotidase activity in T-lymphoblasts. Firstly, if the low K_m soluble 5′-nucleotidase particles in intracellular nucleotide degradation, it seems possible that the altered properties of the enzyme may explain the decreased dephosphorylating activity of these cells. Secondly, the preference of soluble low K_m 5′-nucleotidase for deoxycytidine 5′-monophosphate may facilitate the generation of deoxycytidine. This may explain the selective toxicity of deoxyadenosine for T-lymphoblasts as compared to B-lymphoblasts [7–9, 34, 46]. The soluble low K_m 5′-nucleotidase in B-lymphoblasts may generate levels of deoxycytidine sufficient to saturate deoxycytidine kinase and inhibit deoxyadenosine and deoxyguanosine phosphorylation [47, 48]. In T-lymphoblasts, the reduced and kinetically altered activity of soluble low K_m 5′-nucleotidase and the resultant decreased ability for the removal of excess of nucleoside monophosphates optimizes the conditions for the preferential phosphorylation of accumulated deoxyadenosine and deoxyguanosine to toxic deoxynucleoside triphosphates. Similar metabolic conditions may contribute to the selective cytotoxicity of deoxyribonucleosides for T-cells in immunodeficient patients with adenosine deaminase deficiency or purine nucleoside phosphorylase deficiency [21, 23] or to the selective adenosine release from B cells [11].

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