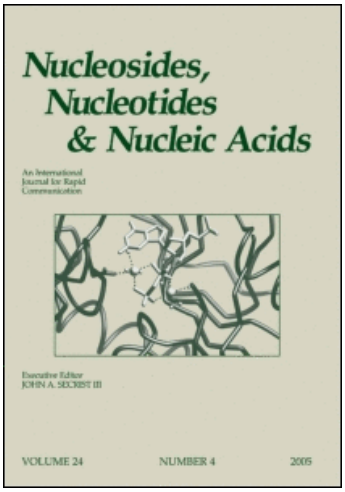

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CHARACTERIZATION OF NUCLEOSIDE PHOSPHOTRANSFERASE FROM *LEISHMANIA TROPICA*

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Abstract: Nucleoside phosphotransferase, a unique purine salvage enzyme of protozoa *Leishmania tropica*, was partially purified. The purified sample was nucleosidase free. With this enzyme preparation, phosphorylation of 14 nucleosides involving 5 antileishmanial purine nucleoside analogs was investigated. Apparent K_m values of the enzyme for the 10 purine nucleosides were in the range 5.7-8.4 μM, and those for the 4 natural pyrimidine nucleosides were 14.0-16.9 μM.

Leishmania tropica, parasitic protozoa causing cutaneous leishmaniasis (1), is incapable of synthesizing purines *de novo* and depends on the host for the source of purines (2). *Leishmania* species has nucleoside phosphotransferase, a unique salvage enzyme that could transfer a phosphate group from various phosphate esters, including *p*-nitrophenyl phosphate, to the 5'-position of nucleosides (3). This enzyme is particularly interesting, since its substrate specificity is not strict and the mammalian hosts do not have this enzyme. This enzyme can phosphorylate nucleoside analogs such as 3'-deoxyinosine (3'-dI) (4), carbocyclic inosine (C-Ino) (5), 3'-deoxy-3'-fluoroinosine (3'-FI) (6, 7), allopurinol riboside (8, 9) and formycin B (10-12) to the corresponding 5'-monophosphates and then the protozoa converts the 5'-monophosphates to 5'-triphosphates with accompanying modification of the bases; e.g., 3'-dI to 3'-deoxyadenosine 5'-triphosphate (3'-dATP), and C-Ino to carbocyclic adenosine 5'-triphosphate (C-ATP) and carbocyclic guanosine 5'-triphosphate (C-GTP). These nucleoside analog 5'-triphosphates are incorporated into nucleic acid and eventually inhibit the synthesis of nucleic acid in *Leishmania* species. Further investigation of the nucleoside phosphotransferase in *Leishmania* species, therefore, will shed light on developing new potent chemotherapeutic agents of leishmaniasis. This enzyme was partially purified from *Leishmania donovani* by Nelson *et al* (8), but the crude enzyme solution contained nucleosidase, which interferes with the enzyme kinetics for nucleoside phospho-

transferase, and they described only the basic kinetics of the enzyme for interconversion of allopurinol riboside.

The present paper describes a procedure for the isolation of nucleoside phosphotransferase from *L. tropica*, nucleosidase free, and discusses the characteristics of the enzyme which phosphorylates nucleosides involving several potent antileishmanial purine nucleoside analogs.

MATERIALS AND METHODS

Materials: 3'-dI and C-Ino were prepared as previously described (4, 5). 3'-FI was obtained from Asahi Glass Corporation (Yokohama, Japan). Hypoxanthine, nucleobases and nucleosides were purchased from Yamasa (Chiba, Japan) and 5-phosphoribosyl-1-pyrophosphate sodium salt (PRPP) and *n*-octyl-β-D-glucopyranoside (n-OGP) from Sigma (St. Louis, MO). Whatman 3 MM cellulose paper was obtained from Whatman (Maidstone, England), thin layer chromatography (TLC) cellulose plate was from Funakoshi (Tokyo, Japan). DEAE Sepharose CL-6B, CM Sepharose CL-6B and Sephacryl S-300 HR and a calibration kit proteins for gel filtration were purchased from Pharmacia (Uppsala, Sweden). Hydroxylapatite HTP and Affi-Gel heparin were products of Bio-Rad (Richmond, CA). Protein concentration was determined by the bicinchoninic acid (BCA) protein assay using bovine serum albumin as a standard (Pierce, Rockford, IL).

Parasites. *Leishmania tropica* promastigotes were obtained from Dr. D. V. Santi (University of California, San Francisco). It was cultured at 25 °C in Dulbecco's modified Eagle medium (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) supplemented with 20% heat-inactivated FBS, 25 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes) (pH 7.4), hemin (5 μg/ml), hypoxanthine (0.7 μg/ml) and gentamycin (25 μg/ml). Cell numbers were counted by using a microcell counter (CC-108, Toa Medical Electronics Co., Kobe, Japan). EC_{50} value refers to the concentration of drug necessary to reduce the growth rate of cells by 50 % of control, as previously described (4, 5).

Preparation of nucleoside phosphotransferase. All steps were carried out at 4 °C. *L. tropica* promastigotes of middle logarithmic phase (2×10^6 cells/ml) were harvested by centrifugation (3,000 × g, 20 min). The 4×10^{10} cells were washed twice with phosphate buffered saline (PBS, pH 7.4) and suspended in 50 mM Hepes buffer (pH 7.4) containing 5 mM $MgSO_4$, 1 mM PRPP, 1 mM dithiothreitol (DTT) and 0.5 mM (*p*-amidinophenyl)-methanesulfonylfluoride hydrochloride (*p*-APMSF). The cell suspension (2 ml) was homogenized by Polytron PTA 7 (Kinematica AG, Littau/Luzern, Switzerland) and

disrupted by ultrasonication (Branson Sonifier 250, Branson Sonic Power Co., Danbury, CT) at an output control 4 and duty cycle (%) 40. The cell lysate was centrifuged at 100,000 × *g* for 1 hr. The supernatant was collected, and the pellet was resuspended in solubilization buffer (the Hepes buffer described above containing 5 % n-OGP) and then incubated for 1 hr. The resuspended solution was centrifuged at 100,000 × *g* for 1 hr. The supernatant was applied to the column chromatography decribed below.

Concentration of enzyme fraction. Concentration of enzyme containing fractions was performed by Centriprep 10 and Centricon 10 (Amicon, Inc. Beverly, MA) at 3,000 × *g* for a desired time.

Desalting by Sephadex G-25 column chromatography. Desalting and buffer change of fractions were performed by passing the solution through a Sephadex G-25 column chromatography. The column (1.6 × 15 cm) was equilibrated with the starting buffer of the next column chromatography and eluted with 60 ml of the buffer at a flow rate of 0.5 ml/min.

CM-Sepharose CL-6B column chromatography. Desalted fractions with enzymatic activity (30 ml) were applied on a CM-Sepharose CL-6B column (1.6 × 5.5 cm) equilibrated with 10 mM potassium phosphate buffer (pH 6.5) containing 1 mM $MgSO_4$, 1 mM DTT, 1mM PRPP, 10 % (v/v) glycerol, 0.8 % n-OGP and 0.5 mM *p*-APMSF. The column was washed with 50 ml of the buffer and subsequently eluted with 200 ml of a linear gradient of 0 to 1.0 M of NaCl in this buffer at a flow rate of 0.5 ml/min. Fractions (28 ml) with enzymatic activity were collected and concentrated. The enzyme fraction was desalted by Sephadex G-25 column chromatography. Fractions (20 ml) containing enzymatic activity were collected.

DEAE-Sepharose CL-6B column chromatography. The fractions were applied on a DEAE-Sepharose CL-6B column (1.6 × 5.5 cm) equilibrated with 10 mM Tris-HCl, pH 8.5 containing 1 mM $MgSO_4$, 1 mM DTT, 1mM PRPP, 10 % (v/v) glycerol, 0.8 % n-OGP and 0.5 mM *p*-APMSF. The column was washed with 50 ml of the same buffer and subsequently eluted with 200 ml of a linear gradient of 0 to 1.0 M of NaCl in this buffer at a flow rate of 0.5 ml/min. Fractions (62 ml) containing enzymatic activity were collected and concentrated. The enzyme fraction was desalted by Sephadex G-25 column chromatography. Fractions (18 ml) with enzymatic activity were collected.

Hydroxylapatite column chromatography. The desalted fractions were applied on a hydroxylapatite column (1.6 × 5.5 cm) equilibrated with 10 mM potassium phosphate

buffer (pH 7.4) containing 1 mM $MgSO_4$, 1 mM DTT, 1mM PRPP, 10 % (v/v) glycerol, 0.8 % n-OGP and 0.5 mM *p*-APMSF. The column was washed with 50 ml of this buffer and subsequently eluted with 200 ml of a linear gradient of 10 to 100 mM of KH_2PO_4 in this buffer at a flow rate of 0.5 ml/min. Fractions (40 ml) with enzymatic activity were collected and concentrated. The enzyme fraction was desalted by Sephadex G-25 column chromatography. Fractions (22 ml) with enzymatic activity were collected.

Affi-Gel heparin affinity column chromatography. The fractions with enzymatic activity were applied on a Affi-Gel heparin affinity column (1.6 × 5.5 cm) equilibrated with 10 mM potassium phosphate buffer (pH 7.4) containing 1 mM $MgSO_4$, 1 mM DTT, 1mM PRPP, 10 % (v/v) glycerol, 0.8 % n-OGP, 0.15 M NaCl and 0.5 mM *p*-APMSF. The column was washed with 50 ml of the same buffer and subsequently eluted with 200 ml of a linear gradient of 0.15 to 2.0 M of NaCl in the buffer at a flow rate of 0.3 ml/min. Fractions (8 ml) with enzymatic activity were collected and concentrated.

Gel filtration of Sephacryl S-300 HR. The concentrated fraction was applied on a Sephacryl S-300 HR gel filtration column of 2.6 × 60 cm, equilibrated with 50 mM Hepes, pH 7.4 containing 5 mM $MgSO_4$, 1 mM DTT, 1mM PRPP, 10 % (v/v) glycerol, 0.8 % n-OGP and 0.5 mM *p*-APMSF. The column was subsequently eluted with same buffer at a flow rate of 1.3 ml/min. Fractions containing enzyme activity were collected and concentrated. Molecular weight of nucleoside phosphotransferase was determined using standard protein of a gel filtration calibration kit (Pharmacia, Uppsala, Sweden).

Assay for nucleoside phosphotransferase. Reaction mixture (20 µl) contained 100 mM Tris-HCl, pH 7.5 with 100 mM *p*-nitrophenyl phosphate, 1 mM C-Ino, 5 mM $MgSO_4$, 1 mM NaF and enzyme solution. A control reaction mixture was prepared in which no enzyme was added. Reaction mixtures were incubated at 37 °C for 1 hr. The reactions were terminated by spotting on a Whatman 3 MM paper. The paper was developed in n-propyl alcohol : conc. NH_4OH : H_2O (6:3:1). The nucleoside and nucleoside 5'-monophosphate in individual reactions well completely separated by this chromatography. The areas corresponding to carbocyclic inosine 5'-monophosphate (C-IMP) were cut out and extracted with 0.01 N HCl. The amount of C-IMP was determined by UV spectrophotometry. One unit of enzyme activity is defined as the amount of enzyme which will catalyze the conversion of 1 µmol of C-Ino to C-IMP per minute at 37 °C.

Nucleosidase assay. Reaction mixtures (20 µl) contained 100 mM Tris-HCl, pH 7.5, 10 mM inosine, 5 mM $MgSO_4$, 1 mM NaF and enzyme solution. Control reaction mixture

did not contain enzyme. Reaction mixture was incubated at 37 °C for 1 hr. The reaction was terminated by spotting on a cellulose plate for TLC. The plate was developed in water. The areas corresponding to hypoxanthine were cut out and extracted with 0.01 N HCl. The amount of hypoxanthine was determined by UV spectrophotometry. One unit of enzyme activity is defined as the amount of enzyme which will catalyze the conversion of 1 μmol of inosine to hypoxanthine per minute at 37 °C. Rf values were as follows : Ino, 0.70 ; Hypoxanthine, 0.55.

Substrate specificity of nucleoside phosphotransferase. Reaction mixtures (20 μl) contained variable concentration of nucleosides and their analogs (5-20 μmol) in 100 mM Tris-HCl (pH 7.5), 100 mM *p*-nitrophenyl phosphate, 5 mM $MgSO_4$ and 1 mM NaF and the enzyme (0.63 units/ml) which had been purified by 5 steps of column chromatography. Assay was performed under conditions used for phosphorylation of C-Ino, as described above. *K*m values for various nucleosides and their analogs were determined by the Hanes-Woolf plotting.

RESULTS

The purification of nucleoside phosphotransferase from *L. tropica* is summarized in TABLE 1. We found that nucleoside phosphotransferase of *L. tropica* exists both in cell membrane and in cytoplasm. The total activity (43.5 unit) and specific activity (8.7 unit/mg of protein) of membrane bound enzyme (5 % n-OGP fraction) were higher than those of cytoplasmic enzyme (supernatant, total activity 34.5 unit; specific activity 2.8 unit/mg of protein) whereas its nucleosidase activity was lower than that of cytoplasmic enzyme. Therefore, we decided to purify the nucleoside phosphotransferase from the cell membrane. Nucleosidase, which could interfere the phosphotransferase assay, was removed completely from the fractions with nucleoside phoshotransferase; the last two steps given in TABLE 1. The nucleosidase-free nucleoside phosphotransferase fraction obtained in the last purification step, Sephacryl S-300 HR gel filtration, showed a total activity and a specific activity of 3.15 unit and 130 unit/mg of protein, respectively (TABLE 1). This specific activity was 30 times higher than that of cell lysate. The molecular weight of the nucleoside phosphotransferase was about 40,000 as determined by Sephacryl S-300 HR gel filtration.

We measured *K*m and *V*max of nucleoside phosphotransferase for various nucleosides and their analogs. The enzyme used in this experiment was the active fraction of Sephacryl S-300 HR gel filtration. The results are shown in FIG. 1 and TABLE 2. FIG. 1 shows *K*m values for C-Ino, 3'-dI, 3'-FI (which are purine nucleoside analogs having selective toxicity against *Leishmania*), and inosine by Hanes-Woolf plotting. In our experiment, as

TABLE 1. Nucleoside phosphotransferase and nucleosidase activities of enzyme-containing fraction in individual purification steps.

Purification step	Protein[a] (mg)	Total activity[b] (unit)	Specific activity (unit/mg of protein)	Nucleosidase[c] activity (unit)
Cell lysate	32	138	4.3	279
Pellet	20	88.0	4.4	122
5% n-OGP fraction	5.0	43.5	8.7	92.7
CM-Sepharose	2.7	28.0	10	63.0
DEAE-Sepharose	0.60	14.4	24	27.1
Hydroxylapatite	0.16	7.52	47	8.54
Affi-Gel heparin	0.070	5.53	79	0
Sephacryl S-300 HR	0.025	3.15	130	0

[a] Protein concentration was assayed by the BCA method.

[b] One unit of enzyme activity is defined as the amount of enzyme which will catalyze the conversion of 1 μmol of carbocyclic inosine to C-IMP per minute at 37 °C.

[c] One unit of enzyme activity is defined as the amount of enzyme which will catalyze the conversion of 1 μmol of inosine to hypoxanthine per minute at 37 °C.

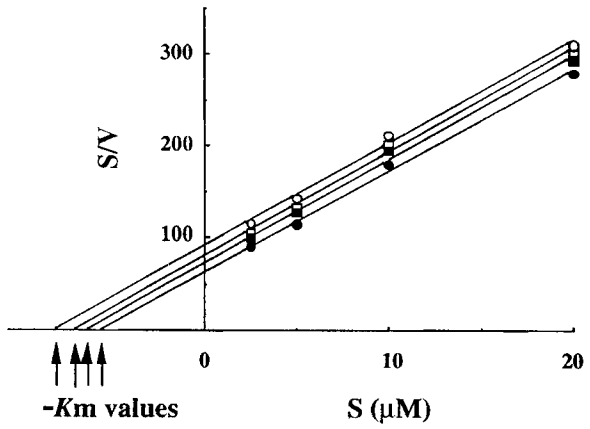

FIG. 1. *K*m value of 3'-dI (○), 3'-FI (□), C-Ino (■) and Inosine (●) by Hanes-Woolf plotting (S/V versus S). V: μmole/min

TABLE 2. *Km* value and *Vmax* of *L. tropica* nucleoside phosphotransferase for nucleosides and their analogs.

Substrate	*Km* value (μM)	*Vmax* (nmol/min/ mg protein)	EC_{50} (M)[a]		
			L. tropica	*L. donovani*	FM3A cell
Inosine	5.9	92	$> 10^4$	$> 10^4$	$> 10^4$
2'-Deoxyinosine	5.7	96	$> 10^4$	$> 10^4$	$> 10^4$
C-Ino	6.5	89	8.3×10^{-8}	1.3×10^{-7}	2.7×10^{-4}
3'-FI	6.6	86	2.3×10^{-7}	1.0×10^{-6}	1.9×10^{-4}
3'-dI	8.4	92	4.4×10^{-7}	1.0×10^{-6}	1.3×10^{-4}
Adenosine	6.1	94	$> 10^4$	$> 10^4$	$> 10^4$
2'-Deoxyadenosine	5.9	98	$> 10^4$	$> 10^4$	1.6×10^{-4}
3'-Deoxyadenosine	7.1	88	3.8×10^{-7}	1.8×10^{-6}	3.3×10^{-6}
Guanosine	6.4	95	$> 10^4$	$> 10^4$	$> 10^4$
2'-Deoxyguanosine	6.3	93	$> 10^4$	$> 10^4$	2.5×10^{-5}
Cytidine	16.9	67	$> 10^4$	$> 10^4$	$> 10^4$
2'-Deoxycytidine	15.3	75	$> 10^4$	$> 10^4$	$>2.0 \times 10^{-3}$
Thymidine	14.0	85	$> 10^4$	$> 10^4$	1.6×10^{-4}
Uridine	16.7	63	$> 10^4$	$> 10^4$	$> 10^4$

[a] EC_{50} value refers to the concentration of drug necessary to reduce the growth rate of cells by 50 % of control (4, 5, 6, 7).

shown in TABLE 2, apparent *Km* values for purine nucleosides and their analogs were similar to each other (5.7-8.4 μM). The *Km* values for pyrimidine nucleosides, which consisted of natural ones only, (14.0-16.9 μM) were about twice higher than those of purine nucleosides. The *Vmax* for purine nucleosides and their analogs were also similar to each other (86-98 nmol/min /mg of protein). The *Vmax* for pyrimidine nucleosides (63-75 nmol/min /mg of protein) were lower than those of purine nucleosides. The *Km* values and *Vmax* of this enzyme for C-Ino, 3'-dI and 3'-FI were similar to normal purine nucleosides (TABLE 2).

DISCUSSION

We purified extensively the nucleoside phosphotransferase from *L. tropica* with 5 steps of column chromatography. The obtained enzyme was nucleosidase-free, and used for investigating phosphorylation for nucleosides involving several purine nucleoside analogs which have potent antileishmanial activity. Since crude enzyme fractions contained nucleosidase, we used C-Ino as the substrate for nucleoside phosphotransferase assay: C-

Ino, having a carbocyclic structure in place of normal sugar, is not cleaved by nucleosidase. Koszalka and Krenitsky have reported that three kinds of nucleosidases isolated from *L. donovani* cleave *N*-ß-glycosidic bond of purine and pyrimidine nucleosides (13). Two kinds of nucleoside-cleaving enzymes have been also recognized in crude promastigote extracts of *L. tropica* (14, 15). One of these was identified as adenosine phosphorylase (EC 2.4.2.1) while the other was termed inosine nucleosidase (EC 3.2.2.2). We were able to remove the nucleosidase from the phoshotransferase fraction by Affi-Gel heparin affinity column chromatography. Although we partially purified the nucleoside phosphotransferase which was able to perform enzyme kinetics on purine and pyrimidine nucleosides, the enzyme was not purified enough to refer to its purity. However, we suppose that the enzyme fractions of Sephacryl S-300 HR were not mixture of several nucleoside phosphotransferase, because pH profile of the enzyme showed bell shape using inosine and thymidine. Optimum pH of the enzyme for phosphate transfer reaction was about 7.5 (data not shown).

As TABLE 2 shows, purine nucleosides were better substrates than pyrimidine nucleosides and, among the purine nucleosides, there was no significant difference in the efficiency of phosphate transfer. 3'-Deoxyinosine analogs, such as 3'-FI and 3'-dI, were cytotoxic for Leishmanial protozoa, although purine analog 2'-deoxyribonucleosides usually have no selective cytotoxicity against *L. tropica* and *L. donovani*. We suppose that the non-toxicity of purine analog 2'-deoxyribonucleosides is due to the cleavage of glycosidic bond by nucleosidase. Indeed, 3'-FI and 3'-dI are only slowly cleaved by *L. tropica* nucleosidase (Shin et al., unpublished results). Krenitsky et al. reported that inosine was completely cleaved by nucleosidase which was contaminating the enzyme solution in their assay system of nucleoside phosphotransferase (16).

Brunngraber and Chargaff (17) had reported about purification and properties of a nucleoside phosphotransferase from carrot. They reported that the pyrimidine nucleosides are better substrates than the purine nucleosides when phenylphosphate serves as the phosphate donor and, in terms of the sugar moiety, 3'-deoxyadenosine is more readily phosphorylated than 2'-deoxyadenosine. These results suggest that nucleoside phosphotransferase from *L. tropica* is different from the enzyme of carrot in substrate specificity. Nelson *et al.* (8) reported that nucleoside phosphotransferase partially purified from *L. donovani* exhibited a *K*m value for allopurinol riboside at 4.6 mM. This *K*m value was about 650 times higher than that of our enzyme for C-Ino. We suppose that the difference of *K*m value between C-Ino and allopurinol riboside may have relevance to the antileishmanial activity of allopurinol riboside for *L.donovani in vitro* (EC_{50} of allopurinol riboside against *L. donovani* is 7×10^{-6} M (18)). From these results, we suppose that an efficient substrate for this enzyme is a good candidate for an antileishmanial agent and that

this enzyme is apparently a key enzyme to provide clues to finding new chemotherapeutic agents of leishmaniasis. More detailed knowledge about characteristics of this enzyme will be helpful to design more potent new chemotherapeutic agents of leishmaniasis.

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