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# Purification and Properties of Inosine-Guanosine Phosphorylase from *Escherichia coli* K-12

GEORGE W. KOSZALKA,* JANEEN VANHOOKE,† STEVEN A. SHORT, AND WILLARD W. HALL
*Wellcome Research Laboratories, Research Triangle Park, North Carolina 27709*

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**A xanthosine-inducible enzyme, inosine-guanosine phosphorylase, has been partially purified from a strain of *Escherichia coli* K-12 lacking the *deo*-encoded purine nucleoside phosphorylase. Inosine-guanosine phosphorylase had a particle weight of 180 kilodaltons and was rapidly inactivated by *p*-chloromercuriphenylsulfonic acid (p-CMB). The enzyme was not protected from inactivation by inosine (Ino), 2′-deoxyinosine (dIno), hypoxanthine (Hyp), $P_i$, or α-D-ribose-1-phosphate (Rib-1-P). Incubating the inactive enzyme with dithiothreitol restored the catalytic activity. Reaction with p-CMB did not affect the particle weight. Inosine-guanosine phosphorylase was more sensitive to thermal inactivation than purine nucleoside phosphorylase. The half-life determined at 45°C between pH 5 and 8 was 5 to 9 min. Phosphate (20 mM) stabilized the enzyme to thermal inactivation, while Ino (1 mM), dIno (1 mM), xanthosine (Xao) (1 mM), Rib-1-P (2 mM), or Hyp (0.05 mM) had no effect. However, Hyp at 1 mM did stabilize the enzyme. In addition, the combination of $P_i$ (20 mM) and Hyp (0.05 mM) stabilized this enzyme to a greater extent than did $P_i$ alone. Apparent activation energies of 11.5 kcal/mol and 7.9 kcal/mol were determined in the phosphorolytic and synthetic direction, respectively. The pH dependence of Ino cleavage or synthesis did not vary between 6 and 8. The substrate specificity, listed in decreasing order of efficiency ($V/K_m$), was: 2′-deoxyguanosine, dIno, guanosine, Xao, Ino, 5′-dIno, and 2′,3′-dideoxyinosine. Inosine-guanosine phosphorylase differed from the *deo* operon-encoded purine nucleoside phosphorylase in that neither adenosine, 2′-deoxyadenosine, nor hypoxanthine arabinoside were substrates or potent inhibitors. Moreover, the *E. coli* inosine-guanosine phosphorylase was antigenically distinct from the purine nucleoside phosphorylase since it did not react with any of 14 monoclonal antisera or a polyvalent antiserum raised against *deo*-encoded purine nucleoside phosphorylase.**

Nucleoside phosphorylases catalyze the reversible phosphorolysis of either purine or pyrimidine nucleosides, yielding pentose-1-phosphate and the nucleobase. Three nucleoside phosphorylases expressed by *Escherichia coli* have been well characterized (10, 12, 20, 24). The *udp* gene product, uridine phosphorylase (EC 2.4.2.3), and the *deoA* gene product, thymidine phosphorylase (EC 2.4.2.4), catalyze the phosphorolysis and synthesis of pyrimidine ribonucleosides and 2′-deoxyribonucleosides, respectively. The third enzyme, purine nucleoside phosphorylase (E.C. 2.4.2.1), encoded by the *deoD* gene of the *deo* operon, cleaves and synthesizes both purine ribonucleosides and 2′-deoxyribonucleosides.

The nucleobase specificity of purine nucleoside phosphorylase is dependent on the source from which it is isolated. Hyp, Gua, Xan, and their respective nucleosides are efficient substrates for most mammalian purine nucleoside phosphorylases, whereas Ade and Ade nucleosides are extremely inefficient substrates (14, 28, 32). The nucleobase specificity of purine nucleoside phosphorylase isolated from *Escherichia coli* is less restricted. Ade, Hyp, Gua, and their nucleosides are all efficient substrates for this enzyme (2, 10). However, Xao is a relatively inefficient substrate. The specificity of the *E. coli* enzyme for the pentosyl moiety is similar to the specificity determined for the mammalian enzyme (2, 28). In the presence of phosphate, both enzymes cleave ribo-, 2′-deoxyribo-, arabino-, 2′,3′-dideoxyribo-, and 5′-deoxyribonucleosides when Hyp is the nucleobase.

In addition to the *deoD*-encoded purine nucleoside phosphorylase, *E. coli* also has the ability to synthesize a second enzyme which catalyzes the cleavage of purine nucleosides (1, 8). This enzyme, the product of the *xapA* gene, is expressed only when the bacteria are grown in a medium containing xanthosine and was therefore named xanthosine phosphorylase. By using a dialyzed cellular extract, the substrate specificity of this xanthosine-induced enzyme was found to differ from that of the purine nucleoside phosphorylase encoded by the *deo* operon (8). Xanthosine phosphorylase did not cleave or synthesize Ado or dAdo, but did catalyze the phosphorolysis of Xao, Ino, and Guo. Thus, the specificity of this *xapA*-encoded nucleoside phosphorylase appeared to be closer to that of mammalian purine nucleoside phosphorylase than to that of the *E. coli deo* operon-derived purine nucleoside phosphorylase.

This study describes the purification and further characterization of *E. coli xapA*-specified nucleoside phosphorylase. Particular emphasis has been placed on the physical, chemical, and catalytic properties of this enzyme. The data presented in this study suggest that the name xanthosine phosphorylase is inappropriate and that the *xapA*-encoded enzyme from *E. coli* should be termed inosine-guanosine phosphorylase.

## MATERIALS AND METHODS

**Abbreviations used.** PIPES, Piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); Ade, adenine; Ado, adenosine; BSA, bovine serum albumin; dAdo, 2′-deoxyadenosine; dGuo, 2′-deoxyguanosine; dIno, 2′-deoxyinosine; 5′-dIno, 5′-deoxyinosine; DTT, dithiothreitol; p-CMB, *para*-chloromercuriphenylsulfonic acid, monosodium salt; FPLC, fast protein liquid chromatography; Gua, guanine; Guo, guanosine; Hyp, hypoxanthine; Ino, inosine; kDa, kilodalton; Rib-1-P, α-D-

* Corresponding author.
† Present address: Department of Biochemistry, University of Wisconsin, Madison, WI 53706.

ribose-1-phosphate; 2'-dRib-1-P, α-D-2'-deoxyribose-1-phosphate; Xan, xanthine; Xao, xanthosine; TLC, thin-layer chromatography.

**Materials.** Xao, hypoxanthine arabinoside, formycin B, bis-Tris propane, PIPES, Rib-1-P, 2'-dRib-1-P, and p-CMB were purchased from Sigma Chemical Co., St. Louis, Mo. All other nucleosides were purchased from Pharmacia Biochemicals Inc., Milwaukee, Wis. Prepunched seven-well agarose plates for Ouchterlony diffusion experiments were from Calbiochem-Behring, La Jolla, Calif. Bovine milk xanthine oxidase (EC 3.5.4.4) was the product of Boehringer Mannheim, Indianapolis, Ind. [8-$^{14}$C]Hyp (42 Ci/mol) and [8-$^{14}$C]Gua hydrochloride (6 Ci/mol) were from New England Nuclear Corp., Boston, Mass. Thin-layer cellulose plates with fluorescent indicator were from Eastman Chemicals, Rochester, N.Y. Ultrapure ammonium sulfate was from Schwarz/Mann, Orangeburg, N.J. Organomercurial agarose gel (Affi-Gel 501) was purchased from Bio-Rad Laboratories, Rockville Center, N.Y. An FPLC system equipped with a Mono Q column (1 by 10 cm) and Superose 12 column (1 by 30 cm) was from Pharmacia, Uppsala, Sweden. Monoclonal antibodies raised against the *E. coli deo*-encoded purine nucleoside phosphorylase were a gift from R. P. Quinn, Wellcome Research Laboratories, Research Triangle Park, N.C.

**Enzyme assays.** All nucleoside phosphorylases were assayed spectrophotometrically at 25°C in 1-cm cuvettes with a Gilford model 250 recording spectrophotometer at a full-scale setting of 0.1 absorbance units. Assay mixtures had a volume of 1 ml.

**Inosine-guanosine phosphorylase.** Reactions were monitored at 293 nm ($\Delta\varepsilon$ = 12.0 $mM^{-1}$ $cm^{-1}$) containing 2.5 mM Ino, 20 mM potassium phosphate, pH 7.0, 0.05 U of desalted xanthine oxidase per ml, and enzyme. One unit of enzyme activity oxidized 1 nmol of Ino per min at 25°C. The substrate velocity of dIno, 5'-dIno, 2',3'-dideoxyinosine, and hypoxanthine arabinoside was also assayed at 293 nm by the addition of xanthine oxidase. The cleavage of Ado and dAdo also utilized the xanthine oxidase-coupled assay and was monitored at 305 nm ($\Delta\varepsilon$ = 15.5 $mM^{-1}$ $cm^{-1}$), as did that of Xao at 292 nm ($\Delta\varepsilon$ = 9.6 $mM^{-1}$ $cm^{-1}$) (21). Reaction mixtures lacking inosine-guanosine phosphorylase served as controls. The cleavage of Guo and dGuo was determined directly at 262.5 nm in the absence of xanthine oxidase ($\Delta\varepsilon$ = −3.60 $mM^{-1}$ $cm^{-1}$).

The synthesis of nucleosides from Rib-1-P and 2'-dRib-1-P was determined spectrophotometrically with reaction mixes containing 0.05 mM Hyp, the appropriate pentose-1-phosphate, and inosine-guanosine phosphorylase in 20 mM bis-Tris propane at pH 7.0. These reactions were monitored at 251 nm ($\Delta\varepsilon$ = 1.60 $mM^{-1}$ $cm^{-1}$).

Kinetic constants for Hyp and Gua could not be determined spectrophotometrically due to the insensitivity of the assay at their low concentration. Consequently, the kinetic constants for these nucleobases were determined with a radiochemical assay. Reactions contained $^{14}$C-purine base, 2 mM Rib-1-P, and enzyme in 10 mM bis-Tris propane, pH 7.0, at 25°C. Inosine-guanosine phosphorylase was diluted for these experiments in buffer containing 2 mg of BSA per ml. Reactions lacking Rib-1-P served as controls. The Hyp reactions were quenched by the addition of an equal volume of 1 mM Hyp and 1 mM Ino before applying a 10-μl sample to a cellulose TLC plate. The Gua reactions were terminated by applying 10 μl of the reaction mix to a TLC plate prespotted with 10 μl of 1 mM Guo and 0.1 mM Gua. All plates were rapidly dried after application of the reaction mixture. Substrates and products were separated by developing in water. The $R_f$ values were as follows: Hyp, 0.63; Ino, 0.84; Gua, 0.36; and Guo, 0.66. The UV-absorbing spots were cut out, and the radioactivity was determined as described previously (21).

Purine nucleoside phosphorylase (EC 2.4.2.1) was assayed by measuring the decrease in absorbance at 250.5 nm ($\Delta\varepsilon$ = −0.95 $mM^{-1}$ $cm^{-1}$) with assay mixtures containing 0.1 mM Ado in 20 mM potassium phosphate buffer, pH 7.4. The purified inosine-guanosine phosphorylase preparation was assayed for purine nucleoside phosphorylase activity by using Ado as the nucleoside substrate at 1 mM and coupling the reaction with xanthine oxidase as described above.

Uridine phosphorylase (EC 2.4.2.3), thymidine phosphorylase (EC 2.4.2.4), and adenosine deaminase (EC 3.5.4.2) were assayed spectrophotometrically as described previously (9).

**Inactivation by p-CMB.** Purified inosine-guanosine phosphorylase (0.25 mg) was incubated with 100 μM p-CMB for 5 min at 25°C in 10 mM bis-Tris propane, pH 7.0. Enzymatic activity was determined spectrophotometrically with Ino as described. The concentration of p-CMB that was present in the assay did not affect the coupling system. Reactivation experiments were performed by adding DTT to a final concentration of 1 mM in the reaction mixture.

**Determination of kinetic constants.** All velocities were calculated from initial linear rates. Constants were determined by directly fitting the data to a hyperbola by the method of Wilkinson (31). $K_i$, $K_m$, and $V_{max}$ values were calculated from data obtained from a minimum of six velocity measurements over a 10-fold range that spanned the $K_m$ value. Inhibition constants expressed as "greater than" values were determined at an inhibitor concentration of 1 mM with Ino at a concentration equal to its $K_m$. All $K_i$ values were determined for the ability of the various compounds to inhibit the phosphorolysis of Ino.

**Molecular weight determination.** Gel filtration chromatography was performed on a Superose 12 column (1 by 30 cm) in 20 mM bis-Tris propane, pH 7.0, with and without 2 mM DTT. Ferritin (440 kDa), aldolase (158 kDa), albumin (67 kDa), ovalbumin (43 kDa), chymotrypsinogin (25 kDa), and RNase A (12 kDa) were the standards used for calibration. Enzyme inactivated with p-CMB prior to chromatography was reactivated after elution from the column by the addition of 1 mM DTT (final concentration) in the fractions.

**Protein determination.** Protein concentrations were determined by using the Coomassie blue method (26). Gamma globulin served as the standard.

**Antibody cross-reactivity and inhibition of catalytic rate.** The ability of inosine-guanosine phosphorylase to react with rabbit polyvalent anti-purine nucleoside phosphorylase serum and with 14 monoclonal murine anti-purine nucleoside phosphorylase antibodies (ascites fluid) was determined by using a double immunodiffusion precipitation assay (23). Immunodiffusion plates were prepared with purified inosine-guanosine phosphorylase (0.25 mg) in the center well and the individual antibody preparations (1.3 mg) in surrounding wells. Plates were incubated at 25°C for 2 days. The plates were visually scanned with the aid of a dissecting microscope and oblique illumination for the appearance of precipitation bands twice a day prior to the conclusion of the 48-h incubation. Purified *E. coli* purine nucleoside phosphorylase (15) served as the positive control.

Catalytic inhibition of inosine-guanosine phosphorylase by the presence of antibodies was determined by using Ino as the substrate. Reaction rates were determined as de-

scribed above. Antibody (100 μg) and enzyme (1.5 μg) in bis-Tris propane, pH 7.0, were incubated at room temperature for 15 min before assay.

**Growth of bacteria.** *E. coli* SS5577 ($F^-$ *thi* Δ*deo*) was grown in 11 liters of minimal medium containing Vogel and Bonner salts (30), supplemented with 0.4% glycerol, 0.2% Casamino Acids, and 5 mg of vitamin $B_1$ per liter as described previously (5) with the addition of 0.15% (wt/vol) Xao. The bacteria were grown in a New Brunswick benchtop fermenter at 37°C. An aeration rate of 400 ml of air per min per liter of medium with a 10-lb/in$^2$ back pressure was maintained during growth. The culture was grown to a density of 4 OD units at 600 nm. The temperature of the culture was lowered to 3°C, and DTT was added to a 1 mM final concentration.

**Enzyme purification.** All operations were performed at 3°C unless otherwise stated.

The entire culture volume was homogenized at 12,000 lb/in$^2$ in a Gaulin laboratory homogenizer equipped with a cell rupture valve at room temperature. Nucleic acids were precipitated with the addition of 1/10 volume of 15% streptomycin sulfate at pH 7. After the solids had settled, the supernatant was pumped off and is referred to as the extract.

Calcium phosphate gel was prepared as described (17) except that the dry weight was 55 mg/ml. An amount of the gel slurry equal to 10% of the extract volume was stirred into the extract.

After several hours without additional stirring, the supernatant was pumped off and diluted to twice its volume with cold deionized water. DE-52 cellulose (slurried in water) was then added until <5% of the enzyme activity remained free in solution. After the resin had settled, the supernatant was discarded and the DE-52 was transferred to a column having a bed volume of 10 by 15 cm. The column was washed with 1 liter of 20 mM potassium phosphate, pH 6.8, containing 1 mM DTT and 0.02% potassium azide (buffer A), followed by 2 liters of buffer A with 0.1 M NaCl. Inosine-guanosine phosphorylase was eluted from the column with a 4-liter linear gradient of 0.1 M to 0.4 M NaCl in buffer A at a flow rate of 1.5 ml/min. Fractions containing the highest levels of enzyme were combined, and protein was precipitated by addition of solid ammonium sulfate to 70% saturation (472 g/liter). The pH was maintained at 6.5 by the addition of 1 N KOH. The precipitate was collected by centrifugation for 15 min at 23,000 × *g*, redissolved in 20 ml of 20 mM bis-Tris propane, pH 6.5, and 0.02% potassium azide (buffer B), and divided into two equal batches. Residual ammonium sulfate and DTT were removed by chromatography through a column (2 by 19.5 cm) of G-25 Sephadex equilibrated with buffer B. The absence of DTT was confirmed by the lack of reaction of the eluate with Ellman reagent (3). These two batches were treated separately for the remaining steps of the purification, but the total activities in Table 1 are treated as single fractions for simplicity.

The desalted protein was applied to a column containing organomercurialagarose (2.5 by 9.5 cm) at a flow rate of 0.2 ml/min. This resin was subsequently washed with buffer B containing 1.0 M NaCl at 2 ml/min. After 3 column volumes, the protein concentration of the eluate was <0.1 mg/ml. The resin was reequilibrated to buffer B, and the inosine-guanosine phosphorylase activity was eluted with 20 mM DTT in buffer B. Fractions with the highest activity were combined and assayed as the Hg-agarose fraction. This material was absorbed onto a Mono Q column (1 by 10 cm) equilibrated with buffer B containing 2 mM DTT at room temperature. The enzyme was eluted with a 330-ml linear 0.10 to 0.25 M KCl gradient in buffer B with 2 mM DTT at a flow rate of 2 ml/min. Fractions were collected, and the appropriate fractions were combined. Inosine-guanosine phosphorylase prepared in this manner was stored at −65°C in small portions. Preparations lost <5% of their activity after 9 months.

TABLE 1. Purification of *E. coli* inosine-guanosine phosphorylase

| Fraction | Total vol (liters) | Total protein (g) | Total activity (U)[a] | Sp act (U/mg) | Recovery (%) |
|---|---|---|---|---|---|
| Extract | 10 | 17.8 | 2,300 | 0.13 | 100 |
| Calcium phosphate | 10.5 | 8.07 | 1,870 | 0.23 | 81 |
| DE-52 | 0.675 | 1.06 | 1,100 | 1.0 | 48 |
| Hg-agarose | 0.100 | 0.175 | 650 | 3.0 | 28 |
| Mono Q | 0.100 | 0.035 | 490 | 14.1 | 21 |

[a] One unit of enzyme activity is defined as the amount of enzyme that cleaves 1 nmol of Ino per min under the assay conditions described.

## RESULTS

**Purification.** Inosine-guanosine phosphorylase was purified 108-fold from a cellular extract prepared from an *E. coli* K-12 strain having a *deo* operon deletion. The enzyme was freed from contaminating activities by a series of absorption, organomercurial, and ion-exchange chromatographies (Table 1). None of the following enzymatic activities were detected in the purified preparation: adenosine deaminase (<0.06 U/mg), thymidine phosphorylase (<0.06 U/mg), uridine phosphorylase (<0.03 U/mg), or purine nucleoside phosphorylase (<0.004 U/mg).

**Physical and chemical properties.** p-CMB (100 μM) completely inactivated the enzyme after 5 min at 25°C and pH 7.0. Ino (1 mM), dIno (1 mM), Hyp (1 mM), Rib-1-P (1 mM), and $P_i$ (20 mM) did not protect against p-CMB inactivation. Treatment of the p-CMB-modified enzyme with 10 mM DTT for 10 min at 25°C restored more than 80% of the original enzyme activity in all cases.

Thermal inactivation studies with the purified enzyme were conducted in the presence of 20 mM DTT over a pH range of 5 to 8 at 45°C. The results (not shown) were consistent with first-order thermal inactivation. Inosine-guanosine phosphorylase was most stable between pH 6 and 7 ($t_{1/2}$, 8 to 9 min) but only slightly less stable at pHs 5 to 6 and 7 to 8 ($t_{1/2}$, 5 to 7 min). Inactivation studies as a function of pH were also conducted at 50°C. The purified enzyme lost 75 to 85% of its activity in the first 3 min in the pH range of 6 to 8 at this temperature.

The ability of substrates to protect purified inosine-guanosine phosphorylase from thermal inactivation is shown in Fig. 1. Under the conditions of inactivation, 1 mM but not 50 μM Hyp provided significant protection from thermal denaturation. The presence of 20 mM potassium phosphate, pH 6.5, and 50 μM Hyp stabilized inosine-guanosine phosphorylase to a greater extent than either component alone. This protection was equal to that observed with 1 mM Hyp. At a concentration of 1 mM, Ino, dIno, or Xao or 2 mM Rib-1-P did not stabilize or destabilize the enzyme activity (data not shown).

The effect of additional protein in the incubation mixture was investigated by comparing, at pH 6.5, the first-order decay constants observed at 45°C. The native enzyme had a $t_{1/2}$ of 9 min. Incubation with BSA present at a final concentration of 1 and 5 mg/ml increased the $t_{1/2}$ for inactivation to 14 and 17 min, respectively.

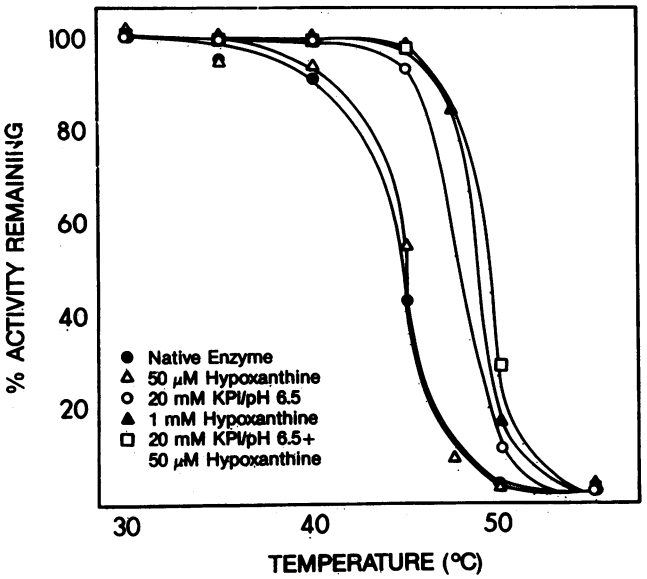


FIG. 1. Thermal stability of inosine-guanosine phosphorylase purified from *E. coli*. Enzyme was incubated in the presence or absence of substrate for 10 min and then stored at 0°C until assayed. Final protein concentration in the incubation mixture was 0.25 mg/ml. Incubation mixtures contained 20 mM bis-Tris propane ($K^+$), 5 mM DTT, substrate, and enzyme. The pH values were calculated from those measured at 25°C by using the relationship $\Delta pK_a/°C = -0.009$ (7). KPi, Potassium phosphate.

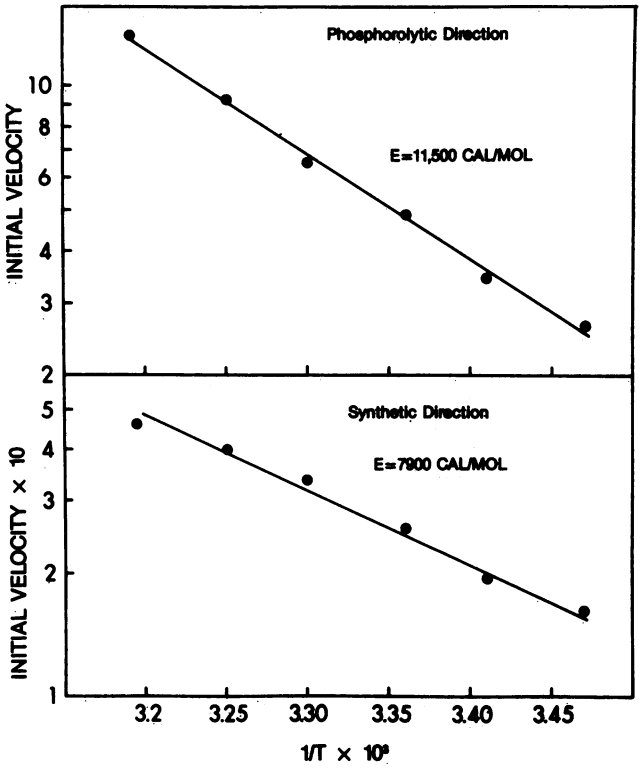


FIG. 2. Arrhenius plots for the synthesis and phosphorolysis of Ino by *E. coli* inosine-guanosine phosphorylase. Reaction mixtures were assayed at 293 nm and contained 2.5 mM Ino, 20 mM potassium phosphate, pH 7.0, 0.05 U of desalted xanthine oxidase in 10 mM potassium-PIPES, pH 7.0, and enzyme ($\Delta\varepsilon$ = 12.0 $mM^{-1}$ $cm^{-1}$). One unit of enzyme activity oxidized 1 nmol of Ino per min at 25°C. Substrate activity in the synthetic direction was determined with 0.05 mM Hyp, 2 mM Rib-1-P, and enzyme in 20 mM bis-Tris propane at pH 7.0. The rate of reaction was determined at 251 nm ($\Delta\varepsilon$ = 1.60 $mM^{-1}$ $cm^{-1}$).

The effect of temperature on the catalytic rate of inosine-guanosine phosphorylase was investigated by determining the apparent activation energies in both the phosphorolytic and synthetic directions (Fig. 2). In the phosphorolytic direction, an apparent activation energy ($E_a'$) of 11.5 kcal/mol was observed. The Arrhenius plot was linear between 15 and 40°C. The $E_a'$ for the synthesis of Ino was less than that observed in the phosphorolytic direction (Fig. 2).

A particle weight of 180 kDa (±20 kDa) for purified inosine-guanosine phosphorylase was estimated by Superose 12 chromatography. Neither p-CMB inactivation of the enzyme prior to chromatography nor inclusion of DTT in the mobile phase of the active enzyme altered the apparent particle weight.

To determine whether any of the monoclonal antibodies or a polyvalent antiserum to the *deo*-encoded purine nucleoside phosphorylase would cross-react with the *xapA*-encoded inosine-guanosine phosphorylase, immunodiffusion plates were loaded with inosine-guanosine phosphorylase in the center well and the various antibody preparations in surrounding wells. No precipitation bands were observed with inosine-guanosine phosphorylase over 2 days. *E. coli* purine nucleoside phosphorylase served as a positive control.

In addition, none of the 14 anti-purine nucleoside phosphorylase murine monoclonal antibodies tested or the anti-purine nucleoside phosphorylase rabbit polyvalent serum altered the rate of Ino phosphorolysis by inosine-guanosine phosphorylase. Five of the 14 monoclonal preparations and the polyvalent antibody preparation significantly inhibited the catalytic rate of the *deo*-encoded purine nucleoside phosphorylase (unpublished data).

**Catalytic properties.** The pH dependence of Ino cleavage or synthesis was not marked between pH 6 and 8. Maximal activity was observed at a pH of 6.6 for phosphorolysis and at a pH of 6.8 in the synthetic direction.

The partially purified enzyme required $P_i$ to catalyze the cleavage of Ino or Xao. In the absence of $P_i$, neither Ino nor Xao was cleaved (<0.003 U/mg). In addition, this enzyme preparation synthesized both Ino and dIno from [$^{14}$C]Hyp and either Rib-1-P or 2'-dRib-1-P, respectively.

The substrate and inhibitor specificities of inosine-guanosine phosphorylase are presented in Table 2. dGuo was the most efficient nucleoside substrate, as judged by the ratio $V_{max}/K_m$, followed by dIno, Guo, Xao, and Ino. Elimination of the 5'-hydroxyl group from Ino or the 3'-hydroxyl group from dIno significantly raised the $K_m$ and lowered the $V_{max}$. Alteration of the 2'-hydroxyl group in Ino from the ribo to the arabino configuration had a profound effect. This analog, hypoxanthine arabinoside, was neither a substrate nor an effective inhibitor. The presence of an amino group in the 6-position also eliminated substrate activity (Ado and dAdo). The purine bases Hyp and Gua had the lowest observed $K_m$ values (3.3 and 4.1 μM, respectively). With both the purine bases and the nucleosides, the presence of an amino group in the 2-position increased the substrate efficiency (Gua versus Hyp). Both of the pentose-1-phosphate analogs had equivalent $K_m$ values, but the $V_{max}$ for 2'-dRib-1-P was threefold greater than for Rib-1-P.

To provide additional evidence that separate enzymes were not responsible for the phosphorolysis of Xao and Ino, apparent inhibition constants ($K_i$) with formycin B at two

TABLE 2. Kinetic constants for inosine-guanosine phosphorylase

| Compound | $K_i$[a] | $K_m$ (μM) | Relative $V_{max}$ (%) | $V_{max}/K_m$ ratio |
|---|---|---|---|---|
| Ino | | 340 ± 8 | 100 | 0.34 |
| Hypoxanthine arabinoside | >3,000 | | <0.01 | |
| 2′-dIno | | 62 ± 4 | 82 | 1.3 |
| 5′-dIno | | 600 ± 50 | 4 | 0.007 |
| 2′,3′-Dideoxyinosine | | 2,600 ± 600 | 3 | 0.001 |
| Xanthosine | | 51 ± 3 | 24 | 0.48 |
| Guo | | 110 ± 8 | 64 | 0.58 |
| dGuo | | 44 ± 4 | 74 | 1.7 |
| Ado | >850 | | <0.02 | |
| dAdo | >850 | | <0.02 | |
| Hyp | | 3.3 ± 0.2 | 15 | 4.6 |
| Gua | | 4.1 ± 0.5 | 49 | 12 |
| Phosphate (potassium) | | 760 ± 20 | 80[c] | |
| Rib-1-P | | 59 ± 5 | 12 | 0.21 |
| 2′-Rib-1-P | | 58 ± 5 | 35 | 0.6 |

[a] All inhibitors showed competive inhibition. Inhibition constants were determined as described in the text.

[b] $V_{max}$ values are expressed relative to that with Ino as the substrate (9.52 U/mg of protein).

[c] Determined at a nonsaturating concentration of Ino (2.5 mM). By assuming Michaelis-Menten kinetics, the estimated relative velocity for phosphate at 2.5 mM is calculated to be 88.

concentrations were determined with Ino and Xao as substrates. The results indicated strict competitive inhibition and equivalent $K_i$ values with both substrates (versus Ino, $K_i$ = 0.30 ± 0.02 mM; versus Xao, $K_i$ = 0.25 ± 0.03 mM). The $V_{max}$ values in both data sets were not significantly different, indicating that the data were consistent for competitive inhibition and inconsistent for other models ($P$ = 0.6 for Ino and 0.4 for Xao) (27).

## DISCUSSION

This study confirms the presence of a second purine nucleoside phosphorylase in *E. coli*. This enzyme, inosine-guanosine phosphorylase, can be induced in an *E. coli* K-12 culture lacking the *deo* operon (8). Inosine-guanosine phosphorylase has been described in crude extracts of *E. coli* (8), but no attempts to purify and further characterize this activity have been reported.

The xanthosine-inducible inosine-guanosine phosphorylase differs in many ways from the *deo* operon-encoded purine nucleoside phosphorylase, most prominently by its lack of either substrate activity or significant inhibition with Ado or dAdo. This contrasts with the rapid rate at which these two nucleosides are catabolized or synthesized by purine nucleoside phosphorylase (12). The substrate specificity of inosine-guanosine phosphorylase observed with nucleosides containing various pentosyl moieties is similar to that observed for the *E. coli* purine nucleoside phosphorylase. Both enzymes cleave ribonucleosides, 2′-deoxy-, 5′-deoxy-, and 2′,3′-dideoxyribonucleosides of Hyp. However, a significant difference was that inosine-guanosine phosphorylase failed to bind or cleave hypoxanthine arabinoside effectively (Table 2). Purine nucleoside phosphorylase is an efficient catalyst for the synthesis of purine arabinonucleosides (4, 16, 29).

Inosine-guanosine phosphorylase also has many physical and chemical properties that are distinct from those of the well-characterized *E. coli* purine nucleoside phosphorylase. Most striking is the susceptibility of inosine-guanosine phosphorylase to thermal denaturation. This enzyme lost 56% of its catalytic activity at 45°C after a 10-min incubation (Fig. 1). Under identical experimental conditions, no loss in enzymatic activity occurred with purine nucleoside phosphorylase (unpublished). In addition, this latter enzyme requires a temperature of 62°C for a 50% loss of catalytic activity within 10 min under similar conditions (15).

It has been widely reported that substrates can enhance the thermal stability of an enzyme. Previously, protection of the *deo*-encoded purine nucleoside phosphorylase from thermal inactivation by various substrates was described (18). All substrates that bind to the free enzyme protect purine nucleoside phosphorylase from thermal inactivation: Ino, $P_i$, Rib-1-P, and 2′-dRib-1-P. Hyp, which by itself does not bind to the free enzyme, does not protect purine nucleoside phosphorylase from thermal inactivation, but Hyp in combination with $P_i$ did protect it. This substrate protection pattern is congruent with the catalytic mechanism proposed for this enzyme (10). The substrate protection pattern for inosine-guanosine phosphorylase is markedly different from that for purine nucleoside phosphorylase. The only substrate that stabilized inosine-guanosine phosphorylase at a concentration approaching its binding constant was $P_i$ (Fig. 1). Neither Ino, Xao, Rib-1-P, nor Hyp (50 μM) protected inosine-guanosine phosphorylase from thermal inactivation. However, as was observed with purine nucleoside phosphorylase, Hyp at 50 μM and $P_i$, the two smallest substrates, when incubated together with the enzyme, stabilized inosine-guanosine phosphorylase to a greater extent than either substrate alone. Kinetic studies have indicated that these two substrates form a dead-end complex with the *E. coli* purine nucleoside phosphorylase (10). It is not known whether Hyp and $P_i$ bind to the free enzyme or form a dead-end complex with inosine-guanosine phosphorylase.

Inosine-guanosine phosphorylase is rapidly inactivated by the sulfhydryl-modifying agent p-CMB, and this inactivation was reversible by addition of DTT. The *E. coli* purine nucleoside phosphorylase is also inactivated by p-CMB but, contrary to inosine-guanosine phosphorylase, dissociates upon inactivation into monomeric subunits (12).

Inosine-guanosine phosphorylase also appears to be antigenically distinct from purine nucleoside phosphorylase. This enzyme failed to form a precipitation band when tested against 14 monoclonal antibodies raised against purine nucleoside phosphorylase or with a polyvalent rabbit anti-purine nucleoside phosphorylase serum. This suggests that inosine-guanosine phosphorylase and purine nucleoside phosphorylase do not have many common antigenic sites. Accordingly, none of these antibody preparations inhibited the catalytic rate of inosine-guanosine phosphorylase. Five of the monoclonal antibodies and the polyvalent antiserum significantly inhibited the catalytic rate of *E. coli* purine nucleoside phosphorylase (unpublished observation).

It has been suggested that the presence of an additional nucleoside phosphorylase in *E. coli* is due to the inability of purine nucleoside phosphorylase to cleave Xao (1). Contrary to the report of Jensen and Nygaard (12), we have observed Xao to be a substrate for a homogeneous preparation of *E. coli* purine nucleoside phosphorylase ($K_m$, 1.6 mM; $V_{max}$, 2.6 μmol/min per mg of protein). This maximal velocity represents 4% of the relative velocity of Ino determined with this preparation of enzyme. It is not known whether this low level of Xao-cleaving activity is sufficient to maintain growth with Xao as the sole carbon source in the absence of the inosine-guanosine phosphorylase gene.

The presence of two purine nucleoside-cleaving activities in the same organism is not unique to *E. coli*. Extracts of

*Leishmania donovani*, *Trypanosoma cruzi*, and *Bacillus subtilis* also contain multiple purine nucleoside-cleaving activities. In all these organisms except *B. subtilis*, one of these activities is hydrolytic rather than phosphorolytic (11, 13, 22, 25). *B. subtilis* has two phosphorylases: one is specific for adenine nucleosides, termed adenosine phosphorylase, and the other cleaves Gua and Hyp nucleosides but not Ado (11). This latter enzyme is termed purine nucleoside phosphorylase, and its specificity differs from that of *E. coli* inosine-guanosine phosphorylase in its lack of activity towards Xao. Another phosphorylase with a substrate specificity similar to that of inosine-guanosine phosphorylase is the human erythrocyte purine nucleoside phosphorylase in that this enzyme does not efficiently cleave Ado nor dAdo but does efficiently cleave the nucleosides of Hyp and Gua (28).

Enzymes are named according to the type of reaction they catalyze and the substrate(s) or substrate type utilized in the reaction (6). The authors who first described inosine-guanosine phosphorylase enzyme termed it xanthosine phosphorylase (8). This name was chosen for two reasons. First, xanthosine was found to be the sole inducer for the synthesis of this protein (8). Second, previous reports indicated that the *deo*-encoded purine nucleoside phosphorylase did not cleave xanthosine (12). However, the name xanthosine phosphorylase suggests that the enzyme is specific for xanthosine. The substrate specificity data indicate that, like mammalian purine nucleoside phosphorylases, this enzyme prefers a 6-oxy group in the purine ring. Since the *deo* operon-encoded purine nucleoside phosphorylase of *E. coli* has an established literature, it would be confusing to call the xanthosine-inducible enzyme purine nucleoside phosphorylase II. Instead, based on the substrate specificity data presented in this study, it is suggested that the nucleoside phosphorylase synthesized by *E. coli* grown in a medium containing xanthosine be termed inosine-guanosine phosphorylase.

## LITERATURE CITED


1. **Buxton, R. S., K. Hammer-Jespersen, and P. Valentin-Hansen.** 1980. A second purine nucleoside phosphorylase in *Escherichia coli* K-12. I. Xanthosine phosphorylase regulatory mutants isolated as secondary-site revertants of a *deoD* mutant. Mol. Gen. Genet. **179**:331–340.
2. **Doskocil, J., and A. Holy.** 1977. Specificity of purine nucleoside phosphorylase from *Escherichia coli*. Collect. Czech. Chem. Commun. **42**:370–383.
3. **Ellman, G. L.** 1959. Tissue sulfhydryl groups. Arch. Biochem. Biophys. **82**:70–77.
4. **Eroshevskaya, L. A., V. N. Barai, A. I. Zinchenko, E. I. Kvasiuk, and I. A. Mikhailopulo.** 1986. Preparative synthesis of the antiviral nucleoside 9-β-D-arabinofuranosyl adenine by using bacterial cells. Antibiot. Med. Biotekhnol. **31**:174–178.
5. **Fischer, M., and S. A. Short.** 1982. Cloning of the *Escherichia coli* K-12 deoxyribonucleoside operon. Gene **17**:291–298.
6. **Florkin, M., and E. H. Stotz.** 1965. Comprehensive biochemistry: enzyme nomenclature, 2nd ed., vol. 13, p. 25–38. Elsevier Publishing Co., New York.
7. **Good, N. E., G. D. Winget, W. Winter, T. N. Connolly, S. Izawa, and R. M. M. Singh.** 1966. Hydrogen ion buffers for biological research. Biochemistry **5**:467–477.
8. **Hammer-Jespersen, K., R. S. Buxton, and T. D. Hansen.** 1980. A second purine nucleoside phosphorylase in *Escherichia coli* K-12. II. Properties of xanthosine phosphorylase and its induction by xanthosine. Mol. Gen. Genet. **179**:341–348.
9. **Ives, D. H., J. P. Durham, and V. S. Tucker.** 1969. Rapid determination of nucleoside kinase and nucleotidase activities with tritium-labled substrates. Anal. Biochem. **28**:192–205.
10. **Jensen, K. F.** 1976. Purine nucleoside phosphorylase from *Salmonella typhimurium* and *Escherichia coli*. Initial velocity kinetics, ligand binding, and reaction mechanism. Eur. J. Biochem. **61**:377–386.
11. **Jensen, K. F.** 1978. Two purine nucleoside phosphorylases in *Bacillus subtilis*. Purification and some properties of the adenosine-specific phosphorylase. Biochim. Biophys. Acta **525**:346–356.
12. **Jensen, K. F., and P. Nygaard.** 1975. Purine nucleoside phosphorylase from *Escherichia coli* and *Salmonella typhimurium*. Purification and some properties. Eur. J. Biochem. **51**:253–265.
13. **Koszalka, G. W., and T. A. Krenitsky.** 1979. Nucleosidases from *Leishmania donovani*. J. Biol. Chem. **254**:8185–8193.
14. **Krenitsky, T. A.** 1967. Purine nucleoside phosphorylase: kinetics, mechanism, and specificity. Mol. Pharmacol. **3**:526–536.
15. **Krenitsky, T. A., G. W. Koszalka, and J. V. Tuttle.** 1981. Purine nucleoside synthesis, an efficient method employing nucleoside phosphorylases. Biochemistry **20**:3615–3621.
16. **Krenitsky, T. A., G. W. Koszalka, J. V. Tuttle, J. L. Rideout, and G. B. Elion.** 1981. An enzymic synthesis of purine D-arabinonucleosides. Carbohydr. Res. **97**:139–146.
17. **Krenitsky, T. A., T. Spector, and W. W. Hall.** 1986. Xanthine oxidase from human liver: purification and characterization. Arch. Biochem. Biophys. **247**:108–119.
18. **Krenitsky, T. A., and J. V. Tuttle.** 1982. Correlation of substrate-stabilization patterns with proposed mechanisms for three nucleoside phosphorylases. Biochim. Biophys. Acta **703**: 247–249.
19. **Krenitsky, T. A., J. V. Tuttle, G. W. Koszalka, I. S. Chen, L. M. Beacham III, J. L. Rideout, and G. B. Elion.** 1976. Deoxycytidine kinase from calf thymus. Substrate and inhibitor specificity. J. Biol. Chem. **251**:4055–4061.
20. **Leer, J. C., K. Hammer-Jespersen, and M. Schwartz.** 1977. Uridine phosphorylase from *Escherichia coli*: physical and chemical characterization. Eur. J. Biochem. **75**:217–224.
21. **Miller, R. L., and D. Lindstead.** 1983. Purine and pyrimidine activities in *Trichomonas vaginalis* extracts. Mol. Biochem. Parasitol. **7**:41–51.
22. **Miller, R. L., C. L. K. Sabourin, and T. A. Krenitsky.** 1987. *Trypanosoma cruzi* adenine nucleoside phosphorylase. Purification and substrate specificity. Biochem. Pharmacol. **36**:553–560.
23. **Ouchterlony, O.** 1959. Diffusion-in-gel methods for immological analysis, p. 1–78. *In* P. Kallos (ed.), Progress in allergy, vol. V. Karger, Basel.
24. **Schwartz, M.** 1971. Thymidine phosphorylase from *Escherichia coli*: properties and kinetics. Eur. J. Biochem. **21**:91–198.
25. **Senesi, S., G. Falcone, U. Mura, F. Sgarrella, and P. L. Ipata.** 1976. A specific adenosine phosphorylase, distinct from purine nucleoside phosphorylase. FEBS Lett. **64**:353–357.
26. **Spector, T.** 1978. Refinement of the Coomassie blue method of protein quantitation: a simple and linear spectrophotometric assay for ≤0.5–50 micrograms of protein. Anal. Biochem. **86**: 142–146.
27. **Spector, T., and G. Hajian.** 1981. Statistical methods to distinguish competitive, noncompetitive and uncompetitive enzyme inhibitors. Anal. Biochem. **115**:403–409.
28. **Stockler, J. D.** 1984. Purine nucleoside phosphorylase; a target for chemotherapy, p. 35–60. *In* R. I. Glazer (ed.), Developments in cancer chemotherapy. CRC Press, Boca Raton, Fla.
29. **Utagawa, T., H. Morisawa, T. Miyoshi, F. Yoshinaga, A. Yamazaki, and K. Mitsugi.** 1980. A novel and simple method for the preparation of adenine arabinoside by bacterial transglycosylation reaction. FEBS Lett. **109**:261–263.
30. **Vogel, H. J., and D. M. Bonner.** 1956. Acetylornithinase of *Escherichia coli*. J. Biol. Chem. **218**:97–106.
31. **Wilkinson, G. N.** 1961. Statistical estimations in enzyme kinetics. Biochem. J. **80**:324–332.
32. **Zimmerman, T. P., N. B. Gersten, A. F. Ross, and R. P. Miech.** 1971. Adenine as substrate for purine nucleoside phosphorylase. Can. J. Biochem. **49**:1050–1054.