Kinetic Properties and Inhibition of Orotidine 5'-Phosphate Decarboxylase

EFFECTS OF SOME ALLOPURINOL METABOLITES ON THE ENZYME

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SUMMARY

The 1- and 7-ribosyl 5'-phosphates of oxipurinol [4,6-dihydroxypyrazolo(3,4-d)pyrimidine] were enzymatically synthesized. These nucleotides, 3-xanthosine 5'-phosphate, and the 1-ribosyl 5'-phosphate of allopurinol [4-hydroxypyrazolo(3,4-d)pyrimidine] were characterized by spectral, chromatographic, and enzymatic procedures. The oxipurinol nucleotides were found to be potent competitive inhibitors of orotidine 5'-phosphate decarboxylase from yeast. This enzyme exhibited bimodal substrate saturation kinetics, the characteristics of which were consistent with the involvement of a single enzyme. Inhibition by nucleotides was also bimodal; thus, two Ki values could be determined for each inhibitor. The most effective inhibitor tested was 1-ribosylxipurinol 5'-phosphate, which had Ki values of 0.02 and 0.003 μM at high (12 to 48 μM) and low (0.5 to 2 μM) substrate concentrations, respectively. The corresponding N-7 derivative had corresponding Ki values of 0.7 and 0.00 μM.

1-Ribosylallopurinol 5'-phosphate, 3-xanthosine 5'-phosphate, and several naturally occurring nucleotides were less inhibitory. Nucleotide inhibitors also stabilized the enzyme against loss of activity at 37°. Inhibition constants determined with the enzyme from rat liver were similar to those from yeast.

The findings furnish direct, quantitative support for the idea that it is the inhibition of orotidine 5'-phosphate decarboxylase by the nucleotides of oxipurinol that is primarily responsible for the increased urinary excretion of orotic acid and orotidine in patients treated with allopurinol.

Allopurinol [4-hydroxypyrazolo(3,4-d)pyrimidine], an inhibitor of xanthine oxidase, is widely used in the treatment of gout and other hyperuricemic conditions (1–3). Patients receiving allopurinol excrete increased amounts of urate and orotidine (4, 5). An early hypothesis (4, 5) proposed that allopurinol increased levels of XMP which, along with the ribonucleotide of orotidine (6), inhibited OMP decarboxylase (EC 4.1.1.23). Fox et al. (7) later reported indirect evidence that the oxidation product of allopurinol, oxipurinol [4,6-dihydroxypyrazolo(3,4-d)pyrimidine] (Fig. 1), was converted to a nucleotide by orotidine 5'-phosphate decarboxylase (EC 2.4.2.10) and that this nucleotide inhibited the OMP decarboxylase. Additional indirect evidence (8, 9) suggested that oxipurinol may be converted to the nucleotides of the two known nucleosides, 1- and 7-ribosylxanthine allopurinol (10), and that both of these nucleotides inhibit OMP decarboxylase.

This report describes the synthesis of the 1- and 7-ribosyl derivatives of oxipurinol and compares their inhibition of yeast OMP decarboxylase with other nucleotides proposed to be important inhibitors of the corresponding mammalian enzyme in vivo. Inhibition constants for the nucleotides of allopurinol and oxipurinol were also determined with rat liver OMP decarboxylase.

EXPERIMENTAL PROCEDURE

Materials

Materials were obtained from the following sources: chromatographically purified Escherichia coli alkaline phosphatase (EC 3.1.3.1), ribothioleotidyl, Mg2+PP-ribose-P, and the sodium salts of UMP, IMP, and XMP from P-L Biochemicals, Milwaukee, Wis.; hydroxylapatite from Clarkson Chemical Co., Williamsport, Pa.; Na4PP-ribose-P, streptomycin sulfate, medium mesh DEAE-cellulose, brewers' bottom yeast, and Crotilus adamanteus 5'-nucleotidase (EC 3.1.3.5) from Sigma Chemical Company, St. Louis, Mo.; DEAE-52 cellulose from Reeves-Angel, Clifton, N. J.; Li2OMP, cyclohexylammonium salt of orotidine, eritroleucovivose glycine, and PMSF from Calbiochem, San Diego, Ca.; enzyme grades of Tris (base), ammonium sulfate, magnesium sulfate, and sucrose from Schwarz-Mann, Orangeburg, N. Y.; amphotolites from LKB, Rockville, Md.; Bio-Gel P-2; (100 to 200 mesh); AG 1-X8 (200 to 400 mesh, chloride form) and ECTEOLA-cellulose (Cellex-E) from Bio-Rad Laboratories, Richmond, Ca.; Sephadex gel from Pharmacia, Piscataway, N. J.; [carboxy-14C]- orotidine 5'-monophosphate, (21 Ci per mole, in 50% ethanol, substantially removed by evaporation to ½ volume with N2) from New England Nuclear, Boston, Mass. [2-14C]uridine 5'-P; PMSF, phenylmethanesulfonyl fluoride; 3 XMP, 3-ribosylxanthine 5'-phosphate; 9-XMP, 9-ribosylxanthine 5'-phosphate; PP-ribose-P, 5- phosphoribosyl 1-pyrophosphate.
monophosphate (27 Ci per mole), [8-14C]inosine 5'-monophosphate (33 Ci per mole), and [2-14C]xanthine (48 Ci per mole) from Schwarz BioResearch, Orangeburg, N. Y.; hydroxide of hyamine, the standard assay conditions described below. Protein concentration was determined by the method of Murphy and Kies (12) with human serum albumin as a protein standard.

**Enzyme Assays**

For all enzyme reactions involved in this study, a unit of activity was defined as that amount of enzyme which would catalyze the formation of 1 nmole of product per min at 37° under the standard assay conditions described below. Protein concentrations were determined by the method of Murphy and Kies (12) with human serum albumin as a protein standard.

**OMP Decarboxylase**—This activity was assayed by either a radiochemical or a spectrophotometric assay. The standard reaction mixture for the radiochemical assay had the following composition: 50 mm Tris-HCl, pH 7.4, 1 mm dithiothreitol, 0.05 mm radioactive OMP, and enzyme in a total volume of 500 μl. Reactions were initiated with either enzyme or with substrate. In the former case (nonpreincubated enzyme) all components except enzyme were incubated at 37° for 5 min and then enzyme (50 μl) was added to start the reaction. In the latter case (preincubated enzyme) all components except OMP, and in some cases inhibitor, were added to the reaction vessels, the vessels were covered with parafilm and incubated for a specific length of time at 37° in a shaking water bath, and the reactions were started by addition of substrate (10 to 50 μl). After incubating at 37° for 5 min, the reactions were stopped with perchloric acid and the released [14C]CO₂ was measured as described by Appel (13). Samples were counted in a Beckman LS-230 scintillation spectrophotometer with an efficiency of 88%.

The optical assay used was essentially that of Lieberman et al. (14). The 1-ml reaction volume contained 50 mm Tris-HCl, pH 7.4, 1 mm dithiothreitol, 0.05 mm OMP, and enzyme. The OMP was added last and the reaction was followed by measuring the decrease of A₂₅₀ nm (Δε = 3950) with a recording spectrophotometer thermostated at 37°.

**Orotate Phosphoribosyltransferase**—The assay for orotate phosphoribosyltransferase was based on the method of Lieberman et al. (14) except that no OMP decarboxylase was added. The 1-ml reaction mixture contained 50 mm Tris-HCl, pH 7.4, 1 mm dithiothreitol, 2 mM MgSO₄, 0.2 mM orotate, 0.5 mM PP-ribose-P, and enzyme. All components except PP-ribose-P were initially added to cuvette, the mixture was incubated for 5 min at 37°, and the reaction was started by addition of 50 μl of PP-ribose-P solution (room temperature). The reaction was followed by measuring the decrease of A₄₉₀ nm (Δε = 18,500) of a reaction solution containing 100 μm glycine, 1 mM MgCl₂, 1 mM ZnCl₂, 6 mM p-nitrophenylphosphate, and 50 μl of appropriately diluted enzyme solution, in a total volume of 3.0 ml at pH 10.5.

**Enzyme Preparations**

**Guanine Phosphoribosyltransferase**—The procedure for the preparation of guanine phosphoribosyltransferase from E. coli was based on purification methods reported previously by Miller et al. (15). Ten grams of the frozen E. coli B cells (16) were suspended in 80 ml of cold 9 mM Tris-HCl, 0.2 mm MgCl₂, pH 7.7 (Buffer A) with a Potter homogenizer and then broken in a cold French Pressure Cell at 17,000 p.s.i. The resulting suspension was centrifuged at 40,000 × g for 30 min at 4°. To 68 ml of the supernatant was added 21 ml of a 10% (w/v) solution of streptomycin sulfate in Buffer A. After stirring at 3° for 30 min, the suspension was centrifuged as described above. The supernatant (87 ml) was applied to a DEAE-cellulose column (3.4 × 44 cm) which had been equilibrated at 24° with Buffer A. The column was eluted at the same temperature with Buffer A containing 0.1 mM KCl until A₂₅₀ nm was less than 0.06 (1200 ml). Elution with Buffer A containing 0.25 mM KCl was then started. After 480 ml of eluate were collected, the enzyme activity was found in the eluate and was present in the next 1280 ml. The enzyme was precipitated at 3° with ammonium sulfate (716 g, 80% saturation), the precipitate was extracted with 19 ml of Buffer A, and the insoluble protein was removed by centrifugation. The soluble protein was desalted on a Sephadex G-25 column (2.5 × 30 cm) equilibrated with Buffer A at 3°. The resultant enzyme preparation (35 ml) contained 5 mg of protein per ml and had a specific activity of 85 units per mg of protein with guanine as the substrate. Activity was determined by the spectrophotometric assay previously described (15).

**Pyrimidine-Ribonucleotide: Pyrophosphate Phosphoribosyltransferase (EC 2.4.2.6)**—This enzyme was partially purified from fresh calf blood through the second step of the procedure described by Hatfield and Wnaggaard (17). The dialyze contained 6 mg of protein per ml with a specific activity of 12 units per mg of protein. The enzyme preparation was stored frozen at -25° until used.

**OMP Decarboxylase**—A partially purified preparation containing both orotate phosphoribosyltransferase and OMP decarboxylase was obtained from P-L Biochemicals as a lyophilized powder, prepared by methanol fractionation of a yeast autolysate (18). It was treated as follows to ensure the removal of small molecules. A solution of 125 mg of the lyophilized powder in 2.5 ml of 5 mM Tris-HCl, pH 7.4 was applied to a Sephadex G-150 column (2.5 × 38 cm) equilibrated with 10 mM Tris-HCl, pH 7.4 at 2°. The column was eluted at a rate of 17 ml per hour. The void volume of the column was 60 ml, while the peak of the OMP decarboxylase activity was eluted at 110 ml and the orotate phosphoribosyltransferase at 116 ml. These elution volumes corresponded approximately to the parti-

When little or no OMP decarboxylase was present, the rate decreased rapidly with time.
The fractions containing greater than 2 units per ml of the decarboxylase were pooled (24 ml), dithiothreitol was added to a concentration of 5 mM, and 17.4 g of ammonium sulfate (95% saturation) were added. The precipitate was collected by centrifugation and dissolved in 2.8 ml of 10 mM Tris-HCl, 1 mM dithiothreitol, pH 7.4. The residual ammonium sulfate was removed by passage through a Sephadex G-200 column (2.5 x 14 cm) equilibrated with 10 mM Tris-HCl, 1 mM dithiothreitol, pH 7.4. This preparation contained 4 mg of protein per ml with a specific activity of 10 units per mg of protein. The enzyme was stable to storage at -25°C. It was used for the experiments shown in all the figures and tables except Fig. 6 and Tables I and IV.

With this OMP decarboxylase preparation, no phosphatase activity was detected when either substrate (standard assay conditions with 0.1 unit of 40-min preincubated enzyme, 1 mM OMP, and 0- to 20-min reactions) or inhibitor (1 mM 7-Oxiz-5'-P, same conditions) was tested. Substrate dephosphorylation was assayed by chromatographic separation, and inhibitor dephosphorylation by monitoring its inhibitory effectiveness (note Table I, Footnote a). To establish the absence of other extraneous reactions of OMP, decarboxylation reactions were allowed to go to completion. These showed 98 ± 2% of the radioactivity of the substrate, OMP, converted to radioactive product, CO2. A linear relationship between initial velocity and the amount of enzyme used was observed in the range from 0.1 to 2 times the amount of enzyme used in the kinetic studies.

OMP decarboxylase was also prepared from yeast by a method which avoided the autolysis step and which included the use of the protease inhibitor, PMSF (19, 20). This was undertaken to reduce the possibility of proteolytic modification of the enzyme. All operations were carried out at 0-4°C. Dried brewers' yeast (2 g) was suspended in 50 ml of 1 mM PMSF in 10% 2-propanol. The suspension was stirred for 30 min, passed through a French Pressure Cell three times at 17,000 p.s.i., and then centrifuged (90,000 x g for 20 min). The supernatant (40 ml) contained 650 units of OMP decarboxylase with a specific activity of 2.3 units per mg of protein. Phosphatase activity was present at this stage of purification (1.6 units per mg of protein). De-

Isoelectric Focusing

An Iseo model 212 analytical density gradient electrophoresis apparatus, thermostated at 4°C, was used for isoelectric focusing. A linear gradient (15 ml) from 300 g of sucrose per liter to 100 g of sucrose per liter containing the sample to be separated and 1% pH 5 to 7 ampholytes was used. The lower electrode solution contained 400 g of sucrose per liter and 1% H2PO4; the upper electrode contained 1% NaOH. All of the above solutions contained 1 mM β-mercaptoethanol and 2 mM Tris-HCl, pH 7.4. A potential of 600 volts was applied for 48 hours and then increased to 1000 volts for 24 hours. The focused material was monitored at 280 nm through a 0.5-cm flow cell as the fractions were collected. The pH value for each fraction was determined at 4°C.

Synthesis of Nucleotide Inhibitors

1-Ribosylxopurinol 5'-Monophosphate—1-Oxi-5'-P was synthesized from oxipurinol and PP-ribose-P using the E. coli guanine phosphoribosyltransferase described above. The reaction mixture (100 ml, 25°C) contained 20 mM Tris-HCl, pH 7.6, 25 mM MgCl2, 3.3 mM oxipurinol, 10 mM Na2PP-ribose-P and 1000 units of enzyme. After 20 hours the reaction was diluted to 2 liters with water and 2 g of AG-1 ion exchange resin was added and stirred for 4 hours during which time the absorbance at 242 nm decreased from 1.66 to 0.48. The resin was poured into a column (1 cm x 4.6 cm) and eluted at a flow rate of 20 ml per hour with a 200-ml linear gradient from 0 to 1.0 M LiCl. The eluate fractions from 30 to 120 ml contained ultraviolet-absorbing material. These fractions were lyophilized, dissolved in 3 ml of H2O and applied to a Bio-Gel P-2 column (2.5 x 85 cm) which had been equilibrated with 1 mM LiCl. Elution with 1 ml LiCl was carried out at a flow rate of 4 ml per hour and fractions of 10 ml were collected. This chromatographic step removed four minor ultraviolet-absorbing contaminants. Fractions 25 to 28 were pooled, lyophilized, and the LiCl was extracted with acetonemethanol (9:1) until a negative chloride test was obtained. This nucleotide and those described below were further characterized (see "Results").

7-Ribosylxopurinol 5'-Monophosphate—This nucleotide was synthesized by using the beef erythrocyte phosphoribosyltransferase preparation described above. The reaction mixture (100 ml, pH 8.5, 37°C) initially contained 2 mM oxipurinol, 5 mM Tris-HCl, 8 mM MgSO4, 1.0 mM Na2PP-ribose-P, and 210 units of enzyme. The reaction was monitored by determining the ratio of A253 nm:A239 nm at pH 9 (initially 0.63 and finally about 1.0). Additional aliquots of PP-ribose-P were added after 5 hours and again at 10 and 20 hours for a total of 0.5 mmole. After 24 hours the reaction mixture was centrifuged to remove a precipitate that had formed. The supernatant and a 10-ml H2O extract of the precipitate were combined and lyophilized. The lyophilized residue was extracted three times (3, 1, and 1 ml) with H2O. These pooled extracts were applied to a Bio-Gel P-2 column (2.5 x 85 cm) that had been equilibrated with 1 mM LiCl. The column was eluted at a flow rate of 14 ml per hour with 1 mM LiCl. Three major and several minor peaks with absorption at 254 nm were eluted. Fractions from the largest major peak with absorbance ratios (A253 nm: A239 nm) of 1.05 ± 0.02 were pooled and diluted to 300 ml. A column (2.5 x 27 cm) was packed with ECTEOLA-cellulose which had been pre-
adjusted to pH 7.0 and then equilibrated at 25° with 1 mM LiCl. The diluted fractions from the P-2 column were then applied at a rate of 44 ml per hour and eluted at the same rate with a 1600-mM linear gradient from 1 to 200 mM LiCl. 7-Oxi-5'-P was eluted between 55 and 70 mM LiCl. Peak fractions with an absorbance ratio of 1.07 to 0.03 were combined, lyophilized, and extracted with acetone-methanol (9:1) to remove LiCl. The residue was twice dissolved in H2O, lyophilized, and extracted with acetone-methanol.

Radioactive 7-Oxi-5'-P was prepared in the same manner with [8-14C]xanthine (0.34 Ci per mole).

3-Ribosylxanthine 5'-Monophosphate—A reaction mixture (20 ml) containing 10 mM Tris-HCl, pH 7.7, 17 mM MgCl2, 2 mM [2-14C]xanthine (0.032 Ci per mole), 3 mM Mg-PP-ribose-P, and 230 units of beef erythrocyte phosphoribosyltransferase (see above) was incubated for 3 hours at 25°. At this point, 20 pmol of the residue was dissolved in H2O, lyophilized, and chromatographed (ascending, Whatman No. 3MM, l-propanol-H2O (1:1)) to remove other salts. The ultraviolet-absorbing spot was extracted from the paper with H2O, diluted with 50 ml of 0.1 M H20, and applied to a Bio-Gel P-2 column (2.5 X 37 cm) which had been equilibrated with 0.01 mM LiCl. After the column was washed with 0.1 mM LiCl at a flow rate of 36 ml per hour. Protein and unreacted xanthine were separated from the nucleotide, which was eluted from 120 to 160 ml of eluate. Fractions containing the nucleotide (first radioactive peak) were lyophilized, extracted with acetone-methanol, and air dried.

RESULTS

Properties of Nucleotide Inhibitors

The oxipurinol nucleotides and 3-XMP were synthesized enzymatically with phosphoribosyltransferases from E. coli or beef erythrocytes and purified by combinations of gel filtration, ion exchange and paper chromatography (see "Experimental Procedure"). Each of these nucleotides, as well as 9-XMP and 1-Alo-5'-P showed a single, symmetrical peak with a typical nucleotide retention time when chromatographed on a Varian LC-1000 high pressure liquid amion exchange chromatograph. The dephosphorylated products (see Table I) of 1-Oxi-5'-P, 7-Oxi-5'-P, and 1-Alo-5'-P all cochromatographed with authentic samples of their respective nucleosides on paper chromatography (ascending, Whatman No. 3MM, Solvent B and C (10)). Other spectral, analytical and chemical properties of the synthesized nucleotides are shown in Table I. The spectral data for 3-XMP at pH 2 were the same as at pH 8.1, which were in close agreement with those reported previously (17, 26).

Properties and Kinetics of OMP Decarboxylase

Effects of Enzyme Incubation—Preincubation of the enzyme in reaction mixtures before addition of OMP resulted in marked decreases of activity (Fig. 2). The decrease in the enzyme activity of OMP decarboxylase by the nucleotides at concentrations that effected about 50% inhibition was eliminated by the 5'-nucleotidase treatment.

These values were identical to those for the authentic nucleosides, and different than the values for allopurinol (0.70) and oxipurinol (0.48).

![Fig. 2. The effect of preincubation at 37° on OMP decarboxylase activity. Standard assay conditions were used and 0.2 units of enzyme was initially present. Velocity is expressed as nanomoles of product formed per 5 min.](http://www.jbc.org/content/253/13/3804/F2)

TABLE I

Properties of nucleotide inhibitors

<table>
<thead>
<tr>
<th>Nucleotide</th>
<th>3-XMP</th>
<th>1-AlO-5'-P</th>
<th>1-Oxi-5'-P</th>
<th>3-Oxi-5'-P</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>2</td>
<td>3</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>( \lambda_{\text{max}} ) (nm)</td>
<td>267</td>
<td>251</td>
<td>234, 249</td>
<td>249</td>
</tr>
<tr>
<td>( \epsilon \times 10^{3} ) (M⁻¹ cm⁻¹)</td>
<td>12</td>
<td>8.55</td>
<td>8.5, 7.6</td>
<td>7.9</td>
</tr>
<tr>
<td>5'-Nucleotidase treatment</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( R_{v} ) value before</td>
<td>0.26</td>
<td>0.25</td>
<td>0.29a</td>
<td>0.26a</td>
</tr>
<tr>
<td>( R_{v} ) value after</td>
<td>0.68</td>
<td>0.62a</td>
<td>0.64a</td>
<td>0.52a</td>
</tr>
</tbody>
</table>

1 Inhibition of OMP decarboxylase by the nucleotides at concentrations that effected about 50% inhibition was eliminated by the 5'-nucleotidase treatment.

2 These values were identical to those for the authentic nucleosides, and different than the values for allopurinol (0.70) and oxipurinol (0.48).
buffer, 10 mM N'-2-ethanesulfonic acid or N, N-bis(2-hydroxyethyl)glycine. Dithiothreitol or 500 mM potassium phosphate buffer, 1 mg per ml human serum albumin, or boiled unchromatographed enzyme.

The rate function in either case was bimodal. Two apparent values for $K_m$ and $V_{max}$ could be calculated: a "high $K_m$" and "high $V_{max}$" at concentrations OMP greater than 7 to 10 mM, and a "low $K_m$" and "low $V_{max}$" at concentrations below 3 to 7 mM. The values of the high $K_m$ (2 μM) were close to those reported in the literature (21-23) for the yeast enzyme (4 to 8 μM).

**Table II**

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Initial velocities</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before preincubation</td>
</tr>
<tr>
<td>No inhibitor with enzyme</td>
<td>5.0</td>
</tr>
<tr>
<td>Inhibitor present with enzyme</td>
<td>2.1</td>
</tr>
<tr>
<td>Inhibitor added with substrate after</td>
<td></td>
</tr>
<tr>
<td>preincubation</td>
<td></td>
</tr>
</tbody>
</table>

**Fig. 3.** Time course of the OMP decarboxylation reaction. Enzyme was either preincubated for 40 min (○, ●) or nonpreincubated (△, ▲) and final reaction mixtures contained either high (open symbols) or low (closed symbols) concentrations of OMP. Standard assay conditions were used for all the reactions except that 20 μM OMP was used as the low concentration of OMP, and 0.4 and 1.9 μM OMP were used as the high concentrations of OMP for the reactions employing preincubated and nonpreincubated enzyme, respectively. The reaction mixture with preincubated enzyme initially contained 0.15 unit of enzyme (therefore, after 40 min they contained about 0.04 unit); those with nonpreincubated enzyme contained 0.06 and 0.04 unit with the high and low OMP concentrations, respectively.

**Fig. 4.** Dependence of apparent enzyme stabilization on inhibitor concentration. Standard assay conditions were used except that the OMP concentration was 0.5 μM. Velocities (units × 106) of reactions containing I-Oxi-δ'-P, at the indicated concentrations, added at the start of the reaction after 40 min of enzyme (initially, 0.13 unit) preincubation (●); inhibitor was present during preincubation as well as during the reactions (○).

Effects of Substrate Concentration—The influence of substrate concentrations on the initial velocity of the reaction, using preincubated and nonpreincubated enzyme, is shown in Fig 5. The rate function in either case was bimodal. Two apparent values for $K_m$ and $V_{max}$ could be calculated: a “high $K_m$” and “high $V_{max}$” at concentrations OMP greater than 7 to 10 μM, and a “low $K_m$” and “low $V_{max}$” at concentrations below 3 to 7 μM. The values of the high $K_m$ (2 μM) were close to those reported in the literature (21-23) for the yeast enzyme (4 to 8 μM). The Hill plot of the data is shown in Fig. 5, inset. At both ex
phosphoribosyltransferase influence on OMP decarboxylase activity, the two enzymes were separated by isoelectric focusing. The double reciprocal plots show the results with both 40 min preincubated (●) and nonpreincubated (○) enzyme. Standard assay conditions with the indicated concentrations of OMP were used. Velocities are expressed as nanomoles of product formed per 5 min. Reaction mixtures with enzyme to be preincubated, initially contained 0.15 unit of enzyme; 0.04 unit was added to the reactions which were to have nonpreincubated enzyme. Inset, the Hill plot of the data from the results with preincubated enzyme.

The kinetic studies were examined to determine if a relationship existed between the apparent biphasic decrease of enzyme activity during preincubation (Fig. 2) and the two values for $K_m$ and $V_{max}$ (Fig. 5). With the preincubated enzyme, the first phase of enzyme decay should have been 85 to 99% complete after 40 min. At that point, if the two decay rates were expressions of two different enzyme activities with different values for $K_m$ and $V_{max}$, one enzyme activity should have been essentially eliminated, and the ratio of high $V_{max}$-low $V_{max}$ should have changed substantially. This ratio (1.3), however, was nearly identical for the nonpreincubated and preincubated enzymes. It therefore appeared that there was no direct relationship between the biphasic decrease of enzyme activity and the bimodal enzyme saturation kinetics.

Several other possible explanations for the occurrence of two $K_m$ values were examined experimentally. The possibility that the enzyme could shift rapidly between associated or dissociated species which had different kinetic constants, and that high OMP concentration promoted a shift to the high $K_m$ (high $V_{max}$) form of the enzyme, was not supported by the experiment shown in Fig. 3 (open circles). The enzyme was preincubated for 40 min without OMP. Assuming the above hypothesis, after this treatment the low $K_m$ (low $V_{max}$) form of the enzyme should have been predominant. The reaction was then initiated with a high concentration of OMP. The time course of reaction did not show any detectable rate acceleration that would be expected if the shift to the high $V_{max}$ enzyme form were taking place. The possibility that such a shift was too rapid to be detected under these conditions was not eliminated.

To test the possibility of multiple enzyme forms or of orotate phosphoribosyltransferase activity on OMP decarboxylase activity, the two enzymes were separated by isoelectric focusing. The kinetic studies were examined to determine if a relationship existed between the apparent biphasic decrease of enzyme activity during preincubation (Fig. 2) and the two values for $K_m$ and $V_{max}$ (Fig. 5). With the preincubated enzyme, the first phase of enzyme decay should have been 85 to 99% complete after 40 min. At that point, if the two decay rates were expressions of two different enzyme activities with different values for $K_m$ and $V_{max}$, one enzyme activity should have been essentially eliminated, and the ratio of high $V_{max}$-low $V_{max}$ should have changed substantially. This ratio (1.3), however, was nearly identical for the nonpreincubated and preincubated enzymes. It therefore appeared that there was no direct relationship between the biphasic decrease of enzyme activity and the bimodal enzyme saturation kinetics.

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Fig. 5. Effect of substrate concentration on the initial velocities of reactions. The double reciprocal plots show the results with both 40-min preincubated (●) and nonpreincubated (○) enzyme. Standard assay conditions with the indicated concentrations of OMP were used. Velocities are expressed as nanomoles of product formed per 5 min. Reaction mixtures with enzyme to be preincubated, initially contained 0.15 unit of enzyme; 0.04 unit was added to the reactions which were to have nonpreincubated enzyme. Inset, the Hill plot of the data from the results with preincubated enzyme.

Fig. 6. Separation of OMP decarboxylase from orotate phosphoribosyltransferase by isoelectric focusing. The protein sample was 4.3 mg of a desalted (Sephadex G-25), commercial preparation containing both orotate phosphoribosyltransferase and OMP decarboxylase from P-L Biochemicals (see "Experimental Procedure," "Enzyme Preparations") Recovery of OMP decarboxylase (○) and orotate phosphoribosyltransferase (●) were 50% and 60%, respectively. Activities of the two enzyme were determined spectrophotometrically (see "Experimental Procedure," "Enzyme Assays") with 100 μl of each of the indicated fractions (0.5 ml), which had been diluted 1:1 with 1× Tris-ICl, pH 7.4 immediately after determination of the pH value (△). Inset, effect of substrate concentration on the velocities of OMP decarboxylation reactions using enzyme from Fraction 23, above, without preincubation (0.02 units, specific activity = 32 units per mg of protein). Other details were as described in Fig. 5.

Yet another possible explanation for the bimodal kinetics of enzyme saturation was considered. Perhaps the enzyme from the yeast was "nicked" in a specific manner by a protease (e.g., during the autolysis of the yeast for the enzyme preparation) such that either two populations of enzyme molecules with different kinetic constants, but nearly identical isoelectric points, or an artifactual single population of enzyme molecules with bimodal kinetics was formed. The kinetics of a yeast enzyme, partially

4 A shoulder of orotate phosphoribosyltransferase activity which focused to the more acidic side of the major peak may correspond to the more anionic minor component of the same enzyme separated with DEAE by Umezawa et al. (21).

4 Since OMP decarboxylase and orotate phosphoribosyltransferase activity were partially separated on Sephadex G-150 (see "Experimental Procedure," the kinetics of OMP decarboxylase were examined using fractions from both sides (24 ml apart) of the peak. $K_m$ values and $V_{max}$ ratios were the same within experimental error, and the kinetics were bimodal.
TABLE III

Competitive inhibitors of yeast OMP decarboxylase

Conditions for the reactions were as described in Fig. 5 (preinubated enzyme) except as noted. The concentrations of OMP were chosen so as to avoid concentrations which would give reciprocal velocities in the curved portion of the bimodal double reciprocal plots. Five to seven concentrations in the range from 12 to 48 μM were used with and without inhibitor (the inhibitor added with the OMP) for the Kᵢ determinations at high OMP concentrations, and a range from 0.5 to 2 μM was used for the determinations at low OMP concentrations. Inhibitor concentrations were adjusted to give approximately 50% inhibition at the lowest concentration of OMP in each range. Kinetic constants were determined from the double reciprocal plots with each range of OMP. All of the nucleotides tested appeared to be competitive inhibitors. The Ki values reported here and in Table IV may be maximal values since the amount of inhibitor bound to the enzyme may not be negligible in comparison to the total amount added.

<table>
<thead>
<tr>
<th>Inhibitora</th>
<th>3-XMP</th>
<th>9-XMP</th>
<th>1-Ala-5'-P</th>
<th>1-Oxi-5'-P</th>
<th>7-Oxi-5'-P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kᵢ value (μM)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>High OMP...</td>
<td>1.0</td>
<td>10.0</td>
<td>3.0</td>
<td>0.02</td>
<td>0.7</td>
</tr>
<tr>
<td>Low OMP...</td>
<td>0.3</td>
<td>1.0</td>
<td>1.0</td>
<td>0.003</td>
<td>0.06</td>
</tr>
<tr>
<td>Kᵢ:Kᵢ&lt;sub&gt;c&lt;/sub&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>High OMP...</td>
<td>0.4</td>
<td>1.0</td>
<td>1.0</td>
<td>0.008</td>
<td>0.3</td>
</tr>
<tr>
<td>Low OMP...</td>
<td>0.7</td>
<td>2.0</td>
<td>2.0</td>
<td>0.007</td>
<td>0.1</td>
</tr>
</tbody>
</table>

The inhibition by the most tightly bound inhibitor, 1-Oxi-5'-P, also appeared to be reversible since prolonged incubation of the enzyme with the inhibitor did not increase its inhibitory effect (Table II). Furthermore, the time course of an inhibited reaction (20 μM OMP, 0.07 μM 1-Oxi-5'-P, 30% inhibition) did not show any detectable increase of inhibition during 20 min.

The Kᵢ values for the purine mononucleotides, IMP, AMP, and GMP, were all determined to be greater than 100 μM at the low OMP range.

Kᵢ values: high OMP (6 determinations), 2.4 ± 0.7 μM; low OMP (6 determinations), 0.45 ± 0.14 μM.

purified at 0-4°C in the presence of the "serine-protease" inhibitor (20), PMSE, and without an autolysis step (see "Experimental Procedure"), were bimodal. Furthermore, the ratio, V<sub>max</sub> high:\V<sub>max</sub> low, was again 1.2.

Inhibition of OMP Decarboxylase

Inhibition constants of purine and purine analogue nucleotides with yeast OMP decarboxylase were determined at both high and low concentration ranges of OMP (Table III and Fig. 7). Previous values (5) reported for 9-XMP and 1-Ala-5'-P (Kᵢ = 0.7 and 0.8 μM, respectively) with human erythrocyte enzyme were determined with low concentrations of OMP (0.2 to 1 μM) and correspond reasonably well with the low Kᵢ values reported here. All of the inhibitors were of the competitive type. The purine nucleotide analogue, 1 Oxi 5'-P, was found to inhibit very strongly. The inhibition constants for this nucleotide were greater than 100-fold lower than those for 9-XMP or those reported in the literature for other naturally occurring nucleotides (13, 17, 21-23, 27). At both high and low concentrations of OMP, the Kᵢ:Kᵢ<sub>c</sub> ratio for 1-Oxi-5'-P was less than 0.01. Less potent, but still strongly inhibitory (Kᵢ:Kᵢ<sub>c</sub> = 0.1-0.3) was 7-Oxi-5'-P. The inhibitory effect of 1-Ala-5'-P was not as pronounced as that of the nucleotides of oxipurinol but was comparable to that of 3-XMP. Table IV shows similar results with

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>1-Ala-5'-P</th>
<th>1-Oxi-5'-P</th>
<th>7-Oxi-5'-P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kᵢ value (μM)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>High OMP...</td>
<td>3</td>
<td>0.002</td>
<td>0.2</td>
</tr>
<tr>
<td>Low OMP...</td>
<td>1</td>
<td>0.0005</td>
<td>0.04</td>
</tr>
<tr>
<td>Kᵢ:Kᵢ&lt;sub&gt;c&lt;/sub&gt;</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>High OMP...</td>
<td>0.7</td>
<td>0.0005</td>
<td>0.04</td>
</tr>
<tr>
<td>Low OMP...</td>
<td>1</td>
<td>0.0005</td>
<td>0.04</td>
</tr>
</tbody>
</table>

* Kᵢ<sub>c</sub> values: high OMP (5 determinations), 4 ± 1 μM; low OMP (5 determinations), 1.0 ± 0.3 μM.
a partially purified rat liver decarboxylase. Bimodal kinetics were observed with this preparation and 1-Oxi-5'-P was, again, the most potent inhibitor of the enzyme. The $K_m$ value for this inhibitor was nearly 10-fold lower than the value with the yeast enzyme, but the value for 7-Oxi-5'-P was only slightly lower and that for 1-Alo-5'-P was about the same.

**Discussion**

A striking property of the OMP decarboxylase was the dependence of the $K_m$ value on the concentration of OMP. Previous studies with yeast OMP decarboxylase (21-23) did not use concentrations of OMP low enough to detect the lower $K_m$ reported here. Appel (13) has reported a second, low $K_m$ for the cow brain enzyme; the high $K_m$ was associated with concentrations of enzyme at about 0.034 units per ml of reaction solution (40 μg of protein per ml), and the low $K_m$ was associated with dilute enzyme concentrations (less than 0.009 units per ml of reaction solution or 10 μg of protein per ml). No OMP concentration range was reported for the low $K_m$ determination. The yeast enzyme, at 0.09 units per ml of reaction mixture (40 μg of protein per ml) and at eight times higher concentrations, showed no dependence of the $K_m$ value on enzyme concentration (see "Results"). It may be that, with the lower OMP concentrations, the instability of the enzyme activity at low concentrations of OMP masks the bimodal kinetics when reaction times longer than 5 min are used.

A second notable characteristic of the enzyme studied here was that it appeared to lose activity rapidly when diluted and incubated at 37°C. The initial phase of this loss of activity was temperature dependent. Binding by substrate or competitive inhibitors eliminated the loss of activity, while dithiothreitol only partially helped to stabilize the activity. Similar effects have been previously reported with the enzyme from yeast and cow brain (13, 21, 22). The effects noted here appeared to be more pronounced, although use of a higher reaction temperature (37°C versus 25°C for the yeast enzyme) and the dilution of the enzyme to concentrations used here appeared to enhance the lability of the enzyme activity.

It is important to note that the bimodal nature of the double reciprocal plots and the decrease of activity with preincubation appeared to be independent of each other. Furthermore, the data argued against the presence of other OMP-metabolizing activities, the possibility of orotate phosphoribosyltransferase-OMP decarboxylase interaction, the participation of multiple enzyme forms, or the possibility of protease action on the enzyme as a cause for the bimodal enzyme saturation kinetics. At the present time it therefore appears probable that with the yeast enzyme the bimodal kinetics is a characteristic of a single OMP decarboxylase. The properties of this enzyme have similarities to other enzymes (28 and references therein, 29-34) which appear to show negative homotropic kinetics. Levitski and Koshland (28) have set forth several characteristics as diagnostic criteria for negative cooperativity in an enzyme. The present enzyme, with a Hill coefficient of less than one (0.4), a biphasic double-reciprocal plot, and an $R_s$ value7 greater than 81 (about 250) meet all of these criteria. Although these criteria are met by OMP decarboxylase, other possibilities such as two noninteracting, unequal sites of catalysis present on the single enzyme molecule have not been eliminated.

As previously noted (see "Results"), the two oxipurinucleotides inhibited yeast OMP decarboxylase more strongly than any naturally occurring inhibitors yet reported. Recently Nelson et al. have shown that these nucleotides, as well as 1-Alo-5'-P, occur in the range of about 0.1 to 1 μm in both the liver and kidney of rats for a few hours after high doses (50 mg per kg, intravenous) of allopurinol. From those concentration values, the values for the inhibition constants reported here, the maximal value of 1 μm for the tissue concentration of OMP in rat liver, it may be concluded that these inhibitors should be capable of inhibiting OMP decarboxylase in vivo, and might, thus, be largely responsible for increased tissue levels and excretion (4, 35, and Footnote 4) of orotate and orotidine in these animals. The possibility that 3-XMP might play a role in OMP decarboxylase inhibition and thus in purine control of de novo UMP synthesis, has been suggested by Fox et al. (7). The $K_i$ values observed here would indicate that this might be possible if 3-XMP were found in tissues near or above the micromolar level. A beef blood enzyme will catalyze the formation of 3-XMP in vitro (17), however, no reports of this nucleotide have been noted in vivo.

Although the nucleotides of oxipurinol were found to bind tightly to the OMP decarboxylase and their presence has been correlated with an excess of orotate or orotidine in the tissues and in the urine, the levels of the uridine nucleotides in these tissues have shown only a small, transitory drop. Similar results have been reported with two other inhibitors of OMP decarboxylase, 6-azauridine 5'-monophosphate (36), and 5-azaacidine 5'-monophosphate (37), in rat liver. The apparent compensation implied by these observations may be attributable to increased levels of OMP decarboxylase as observed in these studies or as observed with patients receiving allopurinol (4, 7, 35, 38). Pinsky and Krooth (39), who earlier observed that 6-azauridine similarly elicited increased levels of orotate phosphoribosyltransferase and OMP decarboxylase in human diploid cell strains grown in culture, suggested that the increased levels of these enzyme activities were due to an accumulation of dihydroorotic acid which then affected the increased levels of activity (40). Other work suggested that the similar response to allopurinol treatment was some sort of enzyme activation (38) or at least partially due to enzyme stabilization (7, 35). The fact that the yeast OMP decarboxylase appears to be stabilized by its inhibitors supports the suggestion that enzyme stabilization may play some role in the in vivo compensation for OMP decarboxylase inhibition in allopurinol-treated patients.

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**References**


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7 The $R_s$ value is defined as the ratio of 90 to 10% saturation velocities (28).

8 The fact that two $K_i$ values can be determined for each inhibitor does not change appreciably with $K_m$ for each inhibitor does not change appreciably with OMP concentration.

9 D. J. Nelson, personal communication.
Kinetic Properties and Inhibition of Orotidine 5’-Phosphate Decarboxylase:
EFFECTS OF SOME ALLOPURINOL METABOLITES ON THE ENZYME
James A. Fyfe, Richard L. Miller and Thomas A. Krenitsky


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