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Guanine Phosphoribosyltransferase from *Escherichia coli*, Specificity and Properties†

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ABSTRACT: The specificity and properties of a novel guanine phosphoribosyltransferase of *Escherichia coli* were studied and compared to those of the hypoxanthine–guanine phosphoribosyltransferase from other sources. The structural requirements for binding of purines to this enzyme were explored by the determination of the K_i values for 100 purines and purine analogs. The most effective binding occurred when the purine contained an oxo or thio group in the 6 position and an amino or hydroxyl group in the 2 position. Unlike the hypoxanthine–guanine phosphoribosyltransferase from other sources, this enzyme bound hypoxanthine 67 times less effectively than guanine and four times less effectively than xanthine. Rates of nucleotide formation from a number of purines and purine analogs were also determined. The enzyme had a pH optimum from 7.4 to 8.2. From secondary double-reciprocal plots derived from an initial velocity analysis, the K_m values were 0.037 mM for guanine and 0.33 mM for 5-phosphoribosyl 1-pyrophosphate. The enzyme was sensitive to inhibition by *p*-chloromercuribenzoate and this inhibition could be reversed by either dithiothreitol or β-mercaptoethanol. The apparent activation energy with guanine as the substrate was 12,800 cal/mol below 23° and 3370 cal/mol above 23°. Using isoelectric focusing, the guanine phosphoribosyltransferase had an apparent p*I* of 5.50 while the p*I* of a second enzyme which was specific for hypoxanthine was 4.8.

Phosphoribosyltransferases (PRTases)[1] which catalyze the condensation of purine bases and PP-ribose-P to form 5′-ribonucleotides are widely distributed in nature. Mammals have two such enzymes. One has specificity for 6-aminopurines (adenosine monophosphate:pyrophosphate phosphoribosyltransferase, EC 2.4.2.7) (Henderson and Gadd, 1968; Krenitsky *et al.*, 1969a) and the other for 6-oxopurines (inosine monophosphate:pyrophosphate phosphoribosyltransferase, EC 2.4.2.8) (Krenitsky *et al.*, 1969b; Miller and Bieber, 1969). The latter enzyme isolated from a number of different sources has been shown to be the same enzyme which converts with similar efficiency both hypoxanthine and guanine to their respective ribonucleotides (Krenitsky *et al.*, 1969b; Miller and Bieber, 1969; Henderson, 1969). In addition to this pair of enzymes, some microorganisms have yet another PRTase which exhibits specificity for xanthine (Kalle and Gots, 1961; Krenitsky *et al.*, 1970). With *E. coli* a different set of three distinct PRTases has been described (Krenitsky *et al.*, 1970). One, like that of other organisms, is specific for 6-aminopurines. Another shows specificity for hypoxanthine and the third acts preferentially on guanine and xanthine. The specificity and properties of the latter enzyme are the subjects of this report.

Materials

Ampholytes were purchased from LKB. Ultra Pure Tris, sucrose, and [2-^{14}C]uracil were purchased from Schwarz/Mann. 4-Hydroxypyrazolo[3,4-*d*][6-^{14}C]pyrimidine and 4,6-dihydroxypyrazolo[3,4-*d*][6-^{14}C]pyrimidine were synthesized in this laboratory (Elion *et al.*, 1966). 2,6-Diamino[8-^{14}C]purine was a gift from Dr. M. Earl Balis of the Sloan-Kettering Institute, New York, N. Y. 6-Mercapto[8-^{14}C]purine was purchased from New England Nuclear Corp. Ecteola-cellulose (Cellex E) was purchased from Bio-Rad Laboratories. Uric acid was purchased from Doughterty Chemical Co.; 8-chloroxanthine and 9-methylguanine from Cyclo Chemical; 1,3-dimethylxanthine from Mallinckrodt Chemical Works; 2,4-dihydroxy-5-aminopyrimidine from Eastman Kodak Co.; guanine, hypoxanthine, xanthine, adenine, purine, 8-bromoguanine, isocytosine, thymine, uracil, 5-bromouracil, 2-amino-4,6-dihydroxypyrimidine, 2-amino-4,6-dimercaptopyrimidine, and 2,4-dihydroxy-6-methylpyrimidine from Sigma Chemical Co. Synthesized in these laboratories were the following: 1-methyl-6-thiopurine and 6-mercapto-9-methylpurine (Elion, 1962); 2-methylamino-6-hydroxypurine (Montgomery and Holum, 1958); 2-dimethylamino-6-hydroxypurine, 2-anilino-6-hydroxypurine, and 2-methylthio-6-hydroxypurine (Elion *et al.*, 1956a); 8-methylguanine (Traube, 1923); 8-methylxanthine (Fischer *et al.*, 1952); 8-hydroxyguanine (Fischer, 1897); 8-mercaptoguanine and 8-methylthioguanine (Elion *et al.*, 1959); 8-aminoguanine (Jones and Robins, 1960); 8-phenylguanine (Elion *et al.*, 1951); 2-acetylamino-6-mercaptopurine (Serkagaku, Kogyo Co., Ltd., 1966); 2-chloro-6-mercaptopurine, 2,6-dimercaptopurine, and 2-methyl-6-mercaptopurine (Hitchings and Elion, 1954); 2-amino-6-mercapto-7-methylpurine (Prasad and Robins, 1957); 2-amino-6-mercapto-9-methylpurine, 2-amino-6-mercapto-9-ethylpurine, 2-amino-6-mercapto-9-*n*-propylpurine, and 2-amino-6-mercapto-9-*n*-butylpurine (Noell and Robins, 1962); 2-amino-6-chloropurine (Hitchings, 1957); 6-carbethoxypurine (Well-

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[1] Abbreviations used are: PP-ribose-P, 5-phosphoribosyl 1-pyrophosphate; *p*-CMB, *p*-chloromercuribenzoate; PRTase, phosphoribosyltransferase; PRT, phosphoribosyl transfer.

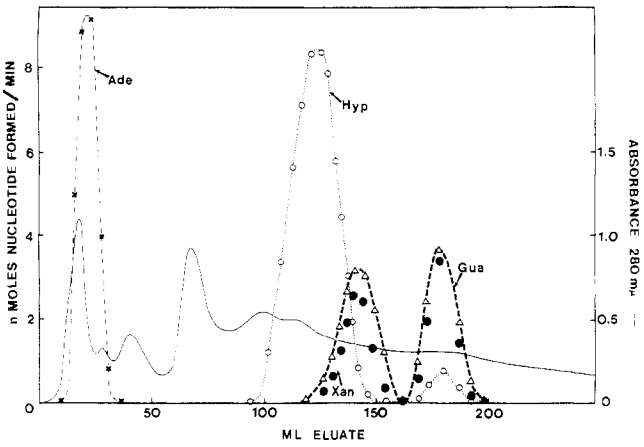

FIGURE 1: Ecteola-cellulose elution profile of the hypoxanthine (Hyp), guanine (Gua), xanthine (Xan), and adenine (Ade) PRT activities of an extract of *E. coli* B. One milliliter of the extract contained 18.4 mg of protein and was capable of catalyzing phosphoribosyl transfer to hypoxanthine, guanine, xanthine, and adenine at rates of 1100, 560, 440, and 660 nmol per min, respectively. Extract (4.0 ml) was applied to a column (1 × 24 cm) which had been previously equilibrated with 9 mM Tris-HCl–0.2 mM $MgSO_4$ (pH 8.2) at 4°. The PRT activities were eluted with a linear gradient of KCl beginning after elution with 50 ml of the equilibrating buffer (see Methods). PRT activities recovered were 80% with hypoxanthine, guanine, and xanthine and 90% with adenine.

come Foundation Ltd., 1958); 2,5-diamino-4,6-dihydroxypyrimidine (Traube, 1893); 2-amino-4-hydroxy-5,6-dimethylpyrimidine (Jaeger, 1891); 2-amino-4-hydroxy-5-carboxypyrimidine (Sorm *et al.*, 1951); 2-amino-4,5-dihydroxypyrimidine (Davoll and Laney, 1956); 2-amino-4-mercapto-6-hydroxypyrimidine and 2-amino-4-mercaptopyrimidine (Koppel *et al.*, 1961); 2-amino-4-mercapto-6-methylpyrimidine (Gabriel and Colman, 1899); 2,4-dihydroxy-5-formamidopyrimidine and 2,4-dihydroxy-5-acetamidopyrimidine (Behrend and Grunwald, 1899); 2,4-dihydroxy-5-carboxamidopyrimidine (Behrend, 1885); xanthopterin (Korte, 1954); isoxanthopterin (Purrman, 1941); 5,7-dihydroxypyrazolo-[4,3-*d*]pyrimidine (Behrend, 1888); 2-amino-4-hydroxypyrido-[2,3-*d*]pyrimidine (Robins and Hitchings, 1955); 2-amino-4-hydroxy-6-nitropyrido[2,3-*d*]pyrimidine (Bernetti *et al.*, 1962); 2-amino-4-hydroxypyrrolo[2,3-*d*]pyrimidine (Wellcome Foundation Ltd., 1959); 2-methyl-5,7-dihydroxythiazolo[4,5-*d*]-pyrimidine (Childress and McKee, 1951); 5-amino-7-hydroxy[1,2,5]thiadiazolo[3,4-*d*]pyrimidine (Shealy *et al.*, 1962); 2-amino-4-mercapto-5,6-cyclopentanopyrimidine (Biglino, 1962); 7-mercaptothiazolo[5,4-*d*]pyrimidine (Elion *et al.*, 1956b); 7-mercapto-*v*-triazolo[4,5-*d*]pyrimidine (Wellcome Foundation Ltd., 1957); and 2-amino-4-hydroxyquinazoline (Kunckell, 1905). The synthesis of 2-amino-4-mercapto-5,6-cyclohexanopyrimidine will be published elsewhere. Other materials were obtained as previously described (Krenitsky *et al.*, 1968, 1969a,b, 1970, 1972).

Methods

Preparation of Guanine Phosphoribosyltransferase (PRTase). Guanine PRTase was partially purified from *Escherichia coli* B by a modified procedure of Krenitsky *et al.* (1970). Prior to Ecteola chromatography, the extract was made 1% with respect to streptomycin by the addition of 5% streptomycin sulfate in 9 mM Tris-HCl–0.2 mM $MgSO_4$ (pH 8.2). The resulting solution, after stirring for 40 min at 4°, was centrifuged at 49,000*g* for 10 min. The streptomycin-treated supernatant was applied to an Ecteola column as previously described (Krenitsky *et al.*, 1970). Elution was carried out with a linear gradient in which the mixing chamber contained 100 ml of 9 mM Tris-HCl–0.2 mM $MgSO_4$ (pH 8.2) and the reservoir contained 100 ml of the same buffer containing 0.3 M KCl. Pooled fractions from the second peak of guanine PRT activity (Figure 1) were used in all following studies. This fraction contained no detectable adenine PRT activity or nucleotide phosphatase activity.[2]

Radioactive Assay. This assay was as previously described (Krenitsky *et al.*, 1970) with the exception that the pH of the Tris-HCl buffer was 7.5 (except where otherwise specified) instead of 7.7 and the $MgSO_4$ concentration was 5.0 mM. Briefly, this assay procedure involves the paper chromatographic separation of the [^{14}C]nucleotide from the [^{14}C]purine base and the determination of the radioactivity of each spot by liquid scintillation.

Spectrophotometric Assays. Spectrophotometric procedures were used only to monitor the Ecteola chromatography and isoelectric focusing of the PRTases. Assay mixtures of a final volume of 1.2 ml contained the following components: adenine, hypoxanthine, guanine, or xanthine, 0.1 mM; Mg_2PP-ribose-P, 1 mM; $MgSO_4$, 5 mM; and Tris-HCl buffer, 0.225 M (pH 9.25 for hypoxanthine and adenine, pH 7.46 for guanine and xanthine). The reactions were started by the addition of enzyme (0.1 ml) and were incubated at 37°. A reaction from which Mg_2PP–ribose-P was omitted was used as a control. Reactions with hypoxanthine as substrate were followed by the increase in absorbance at 245 nm as described by Hill (1970) ($\Delta\epsilon$ = 2500 M^{-1} cm^{-1}). The increase in absorbance at 258 nm was used with adenine as substrate ($\Delta\epsilon$ = 3950 M^{-1} cm^{-1}) and the increase in absorbance at 255 nm was used to assay the conversion of both guanine and xanthine to their mononucleotides ($\Delta\epsilon$ = 4100 and 3600 M^{-1} cm^{-1}, respectively).

Isoelectric Focusing. An Isco Model 212 analytical density gradient electrophoresis apparatus, thermostated at 4°, was used. A linear gradient (16 ml) from 300 to 100 g per l. of sucrose containing enzyme and 1.25% pH 3–10 ampholytes was used. The lower electrode solution contained 400 g/l. of sucrose and 1% H_3PO_4 while the upper electrode contained 1% NaOH. All of the above solutions contained 10 ml of 0.2 M Tris-HCl (pH 7.4)/l. All pH values were measured at 4°.

Results

Enzyme Properties

Behavior on Isoelectric Focusing. Pooled fractions from the second peak of guanine PRT activity eluted from the Ecteola column (Figure 1) were focused in a pH 3–10 gradient. A single symmetrical peak of guanine, xanthine and a small amount of hypoxanthine PRT activity focused at pH 5.50 (Figure 2). The ratios of guanine to xanthine PRT activity and guanine to hypoxanthine PRT activity across this peak were the same as those of the sample prior to focusing. In a separate experiment fractions from the first peak of guanine PRT activity eluted from the Ecteola column (Figure 1) which also contained hypoxanthine-specific PRTase were focused on a pH 3–10

[2] Rechromatography of the second peak of guanine PRT activity under conditions identical with those described resulted in a single symmetrical peak of guanine, xanthine, and hypoxanthine PRT activity. This peak of PRT activity was eluted at the same KCl concentration as that of the second peak of guanine PRT activity in Figure 1. The same relative ratios of guanine to hypoxanthine and guanine to xanthine PRT activities as those prior to rechromatography were observed.

TABLE I: Effects of Various Sulfhydryl Reagents on Aged Enzyme and on Reversal of *p*-CMB Inhibition.[a]

Treatment	Addition	Activity (nmol of GMP Formed per min): Enzyme + Addn	Activity (nmol of GMP Formed per min): *p*-CMB-Treated Enzyme + Addn
(Before storage)	H_2O	1.24	0.48
(After storage at −21° for 95 days)	H_2O	0.20	0.14
(After storage at −21° for 95 days)	Dithiothreitol	1.26	1.22
(After storage at −21° for 95 days)	β-Mercapto-ethanol	0.75	0.67
(After storage at −21° for 95 days)	Reduced glutathione	0.03	0.08

[a] Enzyme which had been stored at −21° in 9 mM Tris-HCl–0.2 mM $MgSO_4$ (pH 7.7 at 25°) was incubated in the presence of 0.1 mM *p*-CMB (where indicated) for 1 min at 38°[b] followed by addition of water or 15 mM dithiothreitol, β-mercaptoethanol, or reduced glutathione. The mixtures were incubated for 10 min and assayed. [b] Maximal inhibition was observed at 10^{-6} M *p*-CMB and always within 1 min after its addition. Concentrations above 10^{-6} M caused no further inhibition.

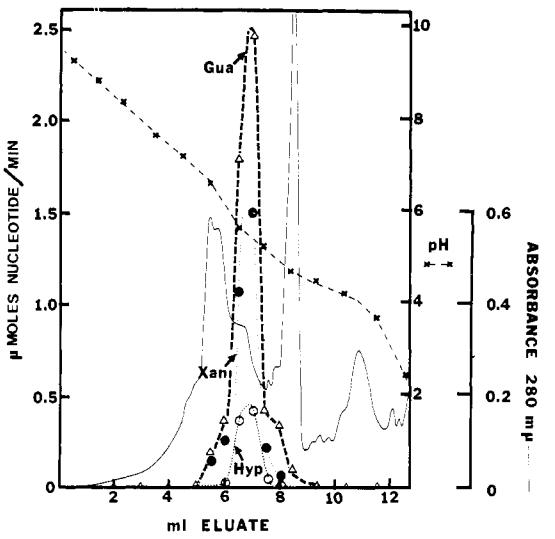

FIGURE 2: Isoelectric focusing profile of the guanine (Gua), xanthine (Xan), and hypoxanthine (Hyp) PRT activities in the second peak of guanine PRT activity from the Ecteola column in Figure 1. Enzyme (8 ml capable of catalyzing phosphoribosyl transfer to guanine, xanthine, and hypoxanthine at rates of 645, 570 and 120 nmol per min, respectively) was applied in a 16-ml sucrose gradient containing 1.25% pH 3–10 ampholytes. A constant voltage of 400 V was applied for 72 hr at 4°. Fractions (0.5 ml) were collected at a flow rate of 0.3 ml/min. PRT activities recovered were 59% with guanine, 56% with xanthine, and 55% with hypoxanthine.

gradient. A peak of guanine, xanthine, and hypoxanthine PRT activity was focused at pH 5.60. This enzyme fraction possessed the same ratios of guanine to xanthine and guanine to hypoxanthine PRT activity as did the focused second peak of guanine PRT activity from the Ecteola column in Figure 1. The hypoxanthine specific PRTase which was present in this fraction was found to focus as a precipitate at pH 4.80. From these data it is evident that the small amount of hypoxanthine PRT activity which was found associated with the guanine PRTase was not a contaminant of the hypoxanthine specific PRTase which focused at pH 4.80.

Stability to Heat. Fractions of enzyme (1.3 ml) in 9 mM Tris-HCl–0.2 mM $MgSO_4$ (pH 7.7 at 25°) were placed in a 60° bath for the appropriate time, withdrawn, and placed directly on ice. Due to the large change in pH of Tris buffers with change in temperature, the pH of the buffer at 60° was 7.1. Heating caused similar fractional reductions in the PRT activities (Figure 3) from the second peak of guanine PRT activity eluted from the Ecteola column (Figure 1) suggesting that all three activities can be attributed to the same enzyme. The hypoxanthine specific PRTase has previously been shown to be less heat stable than the guanine PRTase (Krenitsky *et al.*, 1970).

Stability to pH Change. At 37° in 8 mM Tris–8 mM histidine–8 mM glycine buffer the guanine PRT activity was stable between pH 7.0 and 9.3 for 10 min. However, at pH 4.2 and 11 only 50% of the activity remained after 10 min.

pH Optimum. The pH optimum for guanine PRT activity in Tris-HCl buffer was between 7.4 and 8.2. A gradual decrease in activity was observed on either side of this optimal pH range. At pH 7.0 the activity was 80% maximal while at pH 8.5 it was 70%.

Effect of pH and Magnesium Concentration on K_m Values. A plot of the negative logarithms of the Michaelis constants for guanine (at 1 mM PP-ribose-P) as a function of pH (Figure 4) indicates a small but significant change between pH 7.5 and 8. The possibility that this change in the Michaelis constant for guanine might be reflecting a change in the requirement for magnesium ion was ruled out by an experiment which showed that the reaction velocities at pH values of 7.1, 7.8, and 8.2 showed little or no change at $MgSO_4$ concentrations between 1 and 25 mM. At each pH value a decrease in activity was observed at $MgSO_4$ concentrations above and below this range.

Effect of Temperature on Enzyme Activity. The effect of temperature on the initial rate of guanine PRT activity is shown in Figure 5. The curve is biphasic with a transition at 23°. The calculated activation energies below and above this point are 12,800 and 3,370 cal per mol, respectively. Similar results have been reported for the hypoxanthine–guanine PRTase from yeast (Miller and Bieber, 1969) and Ehrlich ascites cells (Murray, 1967).

Effect of p-CMB and Sulfhydryl Reagents. Storage at −21° in 9 mM Tris-HCl–0.2 mM $MgSO_4$ (pH 7.7 at 25°) caused a gradual decrease in guanine PRT activity. As shown in Table I, treatment of an aged enzyme sample with 15 mM dithiothreitol restored the activity to the level of that of the unaged enzyme.[3] β-Mercaptoethanol was approximately 60% as efficient in restoring activity as was dithiothreitol, whereas treatment with reduced glutathione caused a further decrease in the enzyme activity. A parallel and equal increase in the small amount of hypoxanthine PRT activity was observed in the dithiothreitol-treated sample.

[3] Dithiothreitol treatment had no effect on the Michaelis constant for guanine, whereas it did cause an increase in the maximal velocity relative to the untreated sample.

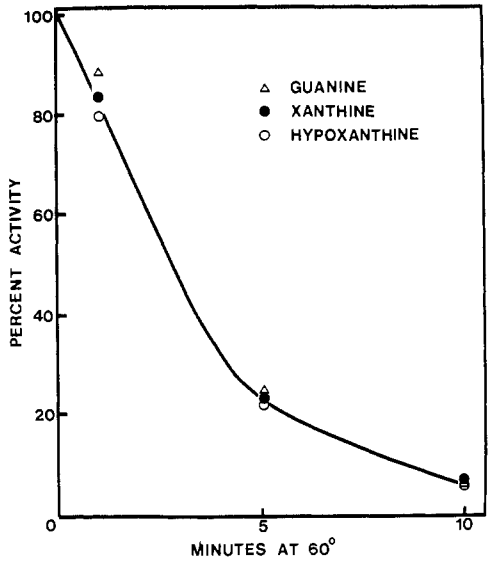

FIGURE 3: Rate of heat inactivation of hypoxanthine (Hyp), guanine (Gua), and xanthine (Xan) PRT activities at 60° in the partially purified guanine PRTase preparation. Enzyme solutions (1.3 ml) contained 1 mg of protein/ml in 9 mM Tris-HCl–0.2 mM $MgSO_4$ (pH 7.7 at 25°). With hypoxanthine, guanine, and xanthine as phosphoribosyl acceptors, the respective activities of the zero time samples were 4, 40, and 37 nmol of nucleotide formed per min per ml of enzyme.

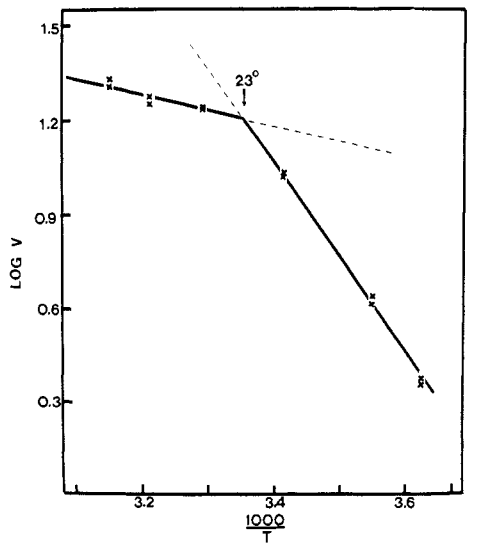

FIGURE 5: Effect of temperature on rate of GMP synthesis. Enzyme assays were conducted at temperatures between 5 and 45°. At each temperature, the Tris buffer was adjusted to pH 7.5. Velocity is nmol of nucleotide formed per min.

Treatment of the enzyme with 0.1 mM *p*-CMB caused a decrease in guanine PRT activity of approximately 70%. As with other PRTases (Hori and Henderson, 1966; Krenitsky and Papaioannou, 1969; Gadd and Henderson, 1970) this enzyme is also protected against *p*-CMB inhibition by PP-ribose-P in the presence of 5 mM $MgSO_4$. The inhibition by *p*-CMB was almost completely reversed by dithiothreitol or β-mercaptoethanol (Table I). The *p*-CMB-inhibited enzyme differed from the untreated enzyme in its heat stability. The *p*-CMB-treated enzyme was completely stable when incubated at 38° for 100 min, whereas a loss in activity of 44% was observed in the absence of *p*-CMB.

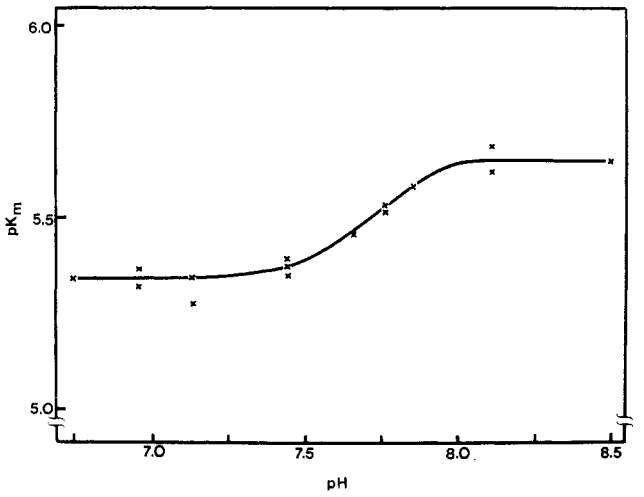

FIGURE 4: Effect of pH on the Michaelis constant for guanine. Assays were run in 0.225 M Tris-HCl–5 mM $MgSO_4$ buffers. Each Michaelis constant value was determined by using seven different concentrations of guanine from 0.2 to 2.3 μM at a Mg_2PP-ribose-P concentration of 1 mM.

Initial Velocity Analysis. As with other purine PRTases (Hori and Henderson, 1966; Henderson *et al.*, 1968; Krenitsky and Papaioannou, 1969), a family of parallel lines was generated when the reciprocal of the initial velocity of GMP formation was plotted as a function of either the reciprocal of the guanine concentration (varied between 2 and 23 μM) or of the PP-ribose-P concentration (varied between 29 and 330 μM) at a constant excess of 5 mM $MgSO_4$. In both cases the reciprocals of the apparent maximal velocities (ordinate intercepts) were a linear function of the reciprocals of the nonvariable substrate concentrations. By extrapolation of these secondary plots, the Michaelis constant for guanine at infinite PP-ribose-P concentration was 0.037 mM, and for PP-ribose-P at infinite guanine concentration was 0.33 mM.

Specificity

Binding of Purines and Purine Analogs. Table II contains inhibition constants for various purines and purine analogs tested as inhibitors of GMP synthesis. Guanine was found to be the most effectively bound of the naturally occurring purines tested. Second was xanthine, which was bound 18 times less effectively than guanine. The apparent Michaelis constant for guanine (3.4 ± 0.7 μM) at 1 mM PP-ribose-P was only slightly higher than its K_i value (Table II). 6-Mercaptopurine and 2-amino-6-mercaptopurine were bound more effectively than the parent oxo compounds while 2-hydroxy-6-mercaptopurine was bound to the same extent as xanthine. Substitutions other than oxo or thio in the 6 position (*e.g.*, hydrogen, amino, methyl, methylamino, methoxy, methylthio, cyano, carboxy, carbethoxy, thiocarboxamido, or chloro), regardless of the substituent in the 2 position, led to a marked decrease in binding.

Binding to the enzyme was strongly influenced by the substituent in the 2 position of the purine ring. With the majority of compounds, the 2-amino derivatives were bound best, followed by 2-hydroxyl > sulfhydryl > hydrogen. Substitutions of one of the hydrogens of the amino group of either guanine

r 2-amino-6-mercaptopurine with a small group (*e.g.*, methyl nd acetyl) decreased binding 20-fold, and bulkier substitu-ons decreased binding even further. Substitution of a di-ıethylamino group resulted in a greater than 100-fold de-rease in binding.

Methylation of ring nitrogen atoms of guanine, 2-amino-6-ıercaptopurine, hypoxanthine, 6-mercaptopurine, and xan-ıine diminished binding drastically. The greatest decreases in inding were with the 2-amino-6-mercaptopurine series of ompounds where decreases of 150-fold (*e.g.*, 7-methyl) to 00-fold (*e.g.*, 1-methyl) were observed relative to the parent ompound.

Substitution in the 8 position had less effect on the binding han substitutions elsewhere on the ring. The 8-amino and 8-ıercapto derivatives of guanine were bound only two to three imes less effectively than guanine. Methylation of the 8-ıercapto group caused a further fivefold decrease in binding /hile a bulkier group in the 8 position (*e.g.*, bromo or phenyl) ed to a drastic decrease in binding.

Of the pyrimidines tested, only 2-amino-4-mercapto-6-ydroxypyrimidine was effectively bound. Of the various con-ensed pyrimidine analogs, the majority of the compounds elected were those which were thought most likely to bind ased on the purine binding data. In general, binding effi-iency decreased in the following order: pteridines > pyrido-2,3-*d*]pyrimidines > pyrrolo[2,3-*d*]pyrimidines > pyrido-3,2-*d*]pyrimidines = quinazoline > *v*-triazolo[4,5-*d*]pyrimi-ines > (1,2,5)thiadiazolo[3,4-*d*]pyrimidines. The 2-amino-4-ydroxy and 2,4-dihydroxy derivatives of pteridine, the 4-ydroxy-6-amino and 4,6-dihydroxy derivatives of pyrazolo-3,4-*d*]pyrimidine, and 2-amino-4-hydroxypyrido[2,3-*d*]pyrimi-ine were bound effectively (*e.g.*, $K_i < 0.08$ mM).

Reaction Velocities. The maximal velocities with several ases were estimated from the initial rates observed at 0.1 mM ^{4}C-labeled base (Table III) and the K_i values (Table II). The urines, guanine, xanthine, adenine, and hypoxanthine, howed high maximal velocities whereas the pyrazolopyrimi-ines reacted slowly. Detectable amounts of mononucleotide /ere not formed from 6-mercaptopurine, 2,6-diaminopurine, r uracil. By the use of the spectral assay of Miller and Bieber 1969), it was shown that, under conditions where guanine /ould be entirely converted to GMP within 10 min, an equiva-ent amount of 2-amino-6-mercaptopurine was less than 20% onverted to its ribonucleotide in 60 min.

Discussion

Partial separation of the guanine and the hypoxanthine PRT ctivities in extracts of *E. coli* B has been previously described y this laboratory (Krenitsky *et al.*, 1970). After Ecteola hromatographic separation of the bulk of the PRT activity oward hypoxanthine from that toward guanine (Figure 1), he second peak of activity[4] toward guanine always had a mall amount of activity toward hypoxanthine. Evidence that nis small amount of hypoxanthine PRT activity is attributable o the broad specificity of the guanine PRTase and not due to ontamination by the hypoxanthine specific PRTase is as ollows. (a) Both isoelectric focusing (Figure 2) and rechroma-ography on Ecteola-cellulose of the guanine PRTase give a ingle symmetrical peak of guanine, xanthine, and hypoxan-ıine PRT activity with ratios of activity for the three sub-strates equivalent to those prior to either isoelectric focusing or rechromatography. (b) Isoelectric focusing has shown that the hypoxanthine specific PRTase has a p*I* of 4.8 whereas the guanine PRTase has a p*I* of 5.5–5.6 and that the second peak of guanine PRT activity from the Ecteola column (Figure 1) possesses no measurable amount of the p*I* 4.8 enzyme. (c) Heat denaturation of the partially purified enzyme preparation showed that all three of the PRT activities present (guanine, xanthine, and the small amount of hypoxanthine) decrease at the same rate (Figure 3). It was previously shown that in crude extracts the hypoxanthine PRTase is much more sensitive to heat denaturation than is the guanine PRTase (Krenitsky *et al.*, 1970). (d) Stability of the three PRT activities to storage at −21° showed that in crude extracts the guanine PRTase was considerably more stable than the bulk of the hypoxanthine PRTase (Krenitsky *et al.*, 1970). However in the partially purified guanine PRTase all three PRT activities decreased at identical rates. (e) Upon reactivation of the partially purified enzyme stored at −21° with dithiothreitol[5] all three of the PRT activities were equally restored. With the guanine PRTase, inhibition constants of hypoxanthine and guanine were 180 and 2.7 μM, respectively (Table II), whereas the corresponding values for the hypoxanthine PRTase were 6 and 1000 μM,[5] thus further substantiating the existence of two distinct 6-oxopurine PRTases. It is also evident from the preceding properties that the small amount of hypoxanthine PRT activity found in the enzyme preparation used in these studies is due to a cross-specificity of the guanine PRTase and is not due to a contamination by the hypoxanthine specific PRTase.

The binding specificity of *E. coli* guanine PRTase for purines and purine analogs (Table II) has both interesting similarities to and differences from that of both the yeast (Miller and Bieber, 1969) and the human erythrocyte hypoxanthine–guanine PRTase (Krenitsky *et al.*, 1969b). All effectively bind 2-amino-substituted purines with an oxo or thio group in the 6 position, but poorly bind all compounds with a 6-amino group. Purines unsubstituted in the 2 position (*e.g.*, hypoxanthine and 6-mercaptopurine) bind much better to the human and yeast enzyme than to the *E. coli* guanine PRTase, whereas those substituted with a 2-hydroxyl group (*e.g.*, xanthine) bind better to the *E. coli* enzyme. Methylation of any of the ring nitrogens drastically decreased binding of the various purine substrates to the *E. coli* enzyme, whereas methyl substitution in the 1 position does not have such a drastic effect on the human hypoxanthine–guanine PRTase (Krenitsky *et al.*, 1969b). This is most evident in the 2-amino-6-mercaptopurine series where 1-methylation caused a 10-fold decrease in binding for the human enzyme (K_i = 0.022 mM) and a 1000-fold decrease for the *E. coli* enzyme (K_i = 0.78 mM) relative to the parent compounds. Although the available data do not allow complete comparison, it appears that binding to the *E coli* enzyme is less affected by methylation on the 2-amino group of guanine or 2-amino-6-mercaptopurine than is the human enzyme.

Koshland and Neet (1968) have described two separate types of enzyme specificity. The first, binding specificity, refers to the effectiveness with which the potential substrate is bound to the active site of the enzyme and the second, kinetic specificity, refers to the specificity in the steps following the binding step. The hypoxanthine–guanine PRTase from human erythrocytes (Krenitsky *et al.*, 1969b) and yeast (Miller and Bieber,

[4] The second peak of guanine PRT activity slowly decreased relative o the first peak upon storage at −21°. The cells used in these studies /ere stored no longer than 6 months.

[5] R. L. Miller, unpublished data.

TABLE II: Inhibition Constants of Purines and Purine Analogs for *E. coli* Guanine PRTase.[a]

Inhibitor	6 Substituent	2 Substituent	Other	$K_i \times 10^3$ M
		Purines		
Purine	H	H		>1
2-Aminopurine	H	NH_2		>1
2-Hydroxypurine[b]	H	OH		0.34
2-Mercaptopurine	H	SH		0.83
Adenine	NH_2	H		1.0
2,6-Diaminopurine	NH_2	NH_2		>1
Hypoxanthine	OH	H		0.18
1-Methylhypoxanthine	O	H	1-CH_3	>1
3-Methylhypoxanthine	O	H	3-CH_3	>1
7-Methylhypoxanthine	OH	H	7-CH_3	>1
9-Methylhypoxanthine	OH	H	9-CH_3	>1
Guanine	OH	NH_2		0.0027[c]
1-Methylguanine	O	NH_2	1-CH_3	0.52
3-Methylguanine	O	NH_2	3-CH_3	0.030
7-Methylguanine	OH	NH_2	7-CH_3	>1
9-Methylguanine	OH	NH_2	9-CH_3	0.47
8-Methylguanine	OH	NH_2	8-CH_3	0.010
8-Aminoguanine	OH	NH_2	8-NH_2	0.0046
8-Hydroxyguanine	OH	NH_2	8-OH	0.031
8-Mercaptoguanine	OH	NH_2	8-SH	0.0074
8-Methylthioguanine	OH	NH_2	8-SCH_3	0.036
8-Bromoguanine	OH	NH_2	8-Br	0.3
8-Phenylguanine	OH	NH_2	8-C_6H_5	>1
2-Methylamino-6-hydroxypurine	OH	$NHCH_3$		0.062
2-Anilino-6-hydroxypurine	OH	NHC_6H_5		0.2
2-Dimethylamino-6-hydroxypurine	OH	$N(CH_3)_2$		0.38
Xanthine	OH	OH		0.048
1-Methylxanthine	O	OH	1-CH_3	0.44
3-Methylxanthine	OH	O	3-CH_3	>1
7-Methylxanthine	OH	OH	7-CH_3	0.41
9-Methylxanthine	OH	OH	9-CH_3	>1
1,3-Dimethylxanthine	O	O	1,3-DiCH_3	0.73
8-Methylxanthine	OH	OH	8-CH_3	0.099
Uric acid	OH	OH	8-OH	>1
8-Chloroxanthine	OH	OH	8-Cl	>1
2-Mercapto-6-hydroxypurine	OH	SH		0.29
2-Methylthio-6-hydroxypurine	OH	SCH_3		0.44
2-Amino-6-methoxypurine	OCH_3	NH_2		>1
6-Mercaptopurine	SH	H		0.038
1-Methyl-6-thiopurine	S	H	1-CH_3	>1
6-Mercapto-7-methylpurine	SH	H	7-CH_3	0.78
6-Mercapto-9-methylpurine	SH	H	9-CH_3	0.84
2-Amino-6-mercaptopurine	SH	NH_2		0.00091
1-Methyl-2-amino-6-thiopurine	S	NH_2	1-CH_3	0.78
2-Amino-6-mercapto-7-methylpurine	SH	NH_2	7-CH_3	0.13
2-Amino-6-mercapto-9-methylpurine	SH	NH_2	9-CH_3	0.46
2-Amino-6-mercapto-9-ethylpurine	SH	NH_2	9-C_2H_5	>1
2-Amino-6-mercapto-9-*n*-propylpurine	SH	NH_2	9-*n*-C_3H_7	>1
2-Amino-6-mercapto-9-*n*-butylpurine	SH	NH_2	9-*n*-C_4H_9	0.6
2-Acetylamino-6-mercaptopurine	SH	$NHCOCH_3$		0.021
2-Hydroxy-6-mercaptopurine	SH	OH		0.046
2,6-Dimercaptopurine	SH	SH		0.23
2-Methyl-6-mercaptopurine	SH	CH_3		0.011
2-Chloro-6-mercaptopurine	SH	Cl		>1
2-Amino-6-methylthiopurine	SCH_3	NH_2		0.48
2-Hydroxy-6-methylthiopurine	SCH_3	OH		0.58
2-Amino-6-methylpurine	CH_3	NH_2		>1
2-Amino-6-cyanopurine	CN	NH_2		>1
6-Carboxypurine	COOH	H		>1
6-Carbethoxypurine	$COOC_2H_5$	H		>1
6-Thiocarboxamidopurine	$CSNH_2$	H		>1
2-Amino-6-chloropurine	Cl	NH_2		0.70

TABLE II (*Continued*)

Inhibitor	Substituents 2	4	5	6	$K_i \times 10^3$ M
Pyrimidines					
Isocytosine	NH_2	OH	H	H	1.0
2-Amino-4,5-dihydroxypyrimidine	NH_2	OH	OH	H	0.1
2-Amino-4,6-dihydroxypyrimidine	NH_2	OH	H	OH	0.4
2,5-Diamino-4,6-dihydroxypyrimidine	NH_2	OH	NH_2	OH	0.2
2-Amino-4-hydroxy-5-carboxypyrimidine	NH_2	OH	COOH	H	>1
2-Amino-4-hydroxy-5,6-dimethylpyrimidine	NH_2	OH	CH_3	CH_3	0.4
2-Amino-4-hydroxy-6-methylpyrimidine	NH_2	OH	H	CH_3	0.3
2-Amino-4-mercaptopyrimidine	NH_2	SH	H	H	0.2
2-Amino-4,6-dimercaptopyrimidine	NH_2	SH	H	SH	0.7
2-Amino-4-mercapto-6-hydroxypyrimidine	NH_2	SH	H	OH	0.034
2-Amino-4-mercapto-6-methylpyrimidine	NH_2	SH	H	CH_3	0.3
Uracil	OH	OH	H	H	>1
5-Bromouracil	OH	OH	Br	H	>1
Thymine	OH	OH	CH_3	H	>1
2,4-Dihydroxy-5-aminopyrimidine	OH	OH	NH_2	H	0.9
2,4-Dihydroxy-5-formamidopyrimidine	OH	OH	NHCHO	H	0.8
2,4-Dihydroxy-5-acetamidopyrimidine	OH	OH	$NHCOCH_3$	H	>1
2,4-Dihydroxy-5-carboxamidopyrimidine	OH	OH	$NHCONH_2$	H	0.4

	Substituents 2	4	6	7	
Pteridines					
2-Amino-4-hydroxypteridine	NH_2	OH	H	H	0.0050
Xanthopterin	NH_2	OH	OH	H	0.040
Isoxanthopterin	NH_2	OH	H	OH	>1
Other Condensed Pyrimidines					
4-Hydroxypyrazolo[3,4-*d*]pyrimidine					>1
4,6-Dihydroxypyrazolo[3,4-*d*]pyrimidine					0.040
4-Hydroxy-6-aminopyrazolo[3,4-*d*]pyrimidine					0.080
5,7-Dihydroxypyrazolo[4,3-*d*]pyrimidine					0.15
2-Amino-4-hydroxypyrido[2,3-*d*]pyrimidine					0.010
2-Amino-4-hydroxy-6-nitropyrido[2,3-*d*]pyrimidine					>1
2,4-Dihydroxypyrido[3,2-*d*]pyrimidine					0.4
2-Amino-4-hydroxypyrrolo[2,3-*d*]pyrimidine					0.2
2-Amino-4-mercapto-5,6-cyclopentanopyrimidine					0.1
2-Amino-4-mercapto-5,6-cyclohexanopyrimidine					0.2
2-Methyl-5,7-dihydroxythiazolo[4,5-*d*]pyrimidine					0.7
7-Mercaptothiazolo[5,4-*d*]pyrimidine					0.3
5-Amino-7-hydroxy[1,2,5]thiadiazolo[3,4-*d*]pyrimidine					>1
7-Mercapto-*v*-triazolo[4,5-*d*]pyrimidine					>1
5-Amino-7-hydroxy-*v*-triazolo[4,5-*d*]pyrimidine					0.5
2-Amino-4-hydroxyquinazoline					0.39

[a] Inhibition constants were calculated from the magnitude of competitive inhibition of each inhibitor with [^{14}C]guanine as substrate. The extent of inhibition was determined at seven concentrations of [^{14}C]guanine ranging from 0.2 to 2.3 μM. Reaction mixture concentrations of Mg_2PP-ribose-P and $MgSO_4$ were kept constant at 1.0 and 5.0 mM, respectively. A single inhibitor concentration ranging from 4 μM for the best inhibitor to >1 mM for the poorest inhibitor was used. Each reaction mixture contained 1 μg of protein. The purines are listed first in order of the 6 substituent, then in order of the 2 substituent. [b] Although some of the compounds listed exist primarily in the oxo or thio form, the substituent is designated OH or SH, respectively, to indicate the availability of a proton. [c] This value was determined by measuring the inhibition of the conversion of [^{14}C]guanine to [^{14}C]GMP by nonradioactive guanine.

1969) and the adenine PRTase from monkey liver (Krenitsky *et al.*, 1969a) have been reported to exhibit differences in binding and kinetic specificities. In the data in Tables II and III are further examples of the difference between the structural requirements for binding and reactivity. Three of the four naturally occurring purines (guanine, xanthine, and

TABLE III: Velocities of Mononucleotide Formation from Purines and Purine Analogs.[a]

^{14}C-Labeled Base	nmol of Nucleotide Formed per min	
	Obsd Rates	Estd[b] Max. Velocities
Guanine	94	97
Xanthine	69	97
Hypoxanthine	18	53
Adenine	9.1	97
4,6-Dihydroxypyrazolo-[3,4-*d*]pyrimidine	0.52	0.72
4-Hydroxypyrazolo-[3,4-*d*]pyrimidine	0.18	2.7[c]
6-Mercaptopurine	<0.02	<0.03
2,6-Diaminopurine	<0.02	
Uracil	<0.02	

[a] Reaction mixture concentrations of ^{14}C-labeled base and PP-ribose-P were 0.1 and 1 mM, respectively. Other assay conditions are as described under Methods. [b] Maximal velocities were calculated from

$$V = v\left(\frac{K_m}{[S]} + 1\right)$$

K_m was assumed equal to K_i (Table II). [c] The K_i value for 4-hydroxypyrazolo[3,4-*d*]pyrimidine was 1.4 mM.

adenine) show the same maximal velocities with the guanine PRTase of *E. coli* B, although there is a range of 370-fold in their binding constants. Adenine, which is bound very poorly, reacts rapidly once bound. Substitution of a mercapto group for the 6-hydroxyl group of hypoxanthine causes a 5-fold increase in binding, but an 1800-fold decrease in reaction rate. The same observation was made for guanine and its 6-mercapto derivative in a semiquantitative fashion (see Results). Thus, although the replacement of a 6-hydroxyl group by a mercapto group causes an increase in binding, the guanine PRTase of *E. coli* B converts 6-mercapto-substituted purines into their respective 5′-nucleotides at very low rates relative to their oxo counterparts. These observations have also been made for the hypoxanthine PRTase from brewers yeast (Miller and Bieber, 1969). In contrast, the rate of reaction of 6-mercaptopurine with the human enzyme is only slightly slower than that of hypoxanthine (Krenitsky *et al.*, 1969b).

For both the *E. coli* guanine PRTase (Table III) and the human erythrocyte hypoxanthine–guanine PRTase (Krenitsky *et al.*, 1969b), guanine was found to have a somewhat higher maximal velocity than hypoxanthine. Relative to guanine the *E. coli* enzyme reacts with xanthine and adenine at rates 69- and 140-fold faster, respectively, than the human erythrocyte enzyme while it reacts with 6-mercaptopurine and 4-hydroxypyrazolo[3,4-*d*]pyrimidine at decreased rates of 2400-fold and 4-fold, respectively.

In light of the recently reported existence of inosine kinase in *E. coli* (Allan and Bennett, 1971) and the existence of purine PRTases which have different specificities as compared to those of mammalian sources, it is becoming increasingly apparent that the salvage pathways of purine metabolism in this organism are significantly different from those of mammalian systems.

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Subunit Structure of the Thermophilic Aminopeptidase I from *Bacillus stearothermophilus*†

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ABSTRACT: The subunit structure of the high molecular weight aminopeptidase I from *Bacillus stearothermophilus* was investigated. The enzyme contains two different subunit types in the ratio 2:1. The amino-terminal sequences of the subunits were determined. The predominant component has the sequence H-Ala-Lys-Leu-Asp-Glu-Thr-Leu-Thr-Met-Leu-Lys-Ala-Leu-Thr-Asp-Ala-. The minor component has the sequence H-Met-Asn-Glu-Glu-Thr-Leu-Gln-. The partially purified aminopeptidase preparation also contains two minor components, both of which have the same two subunit types as the major component but in different relative amounts, *i.e.*, 1:1 and most probably 5:1. Only the predominant type of subunit is necessary for aminopeptidase activity since a fully active aminopeptidase containing just this subunit can be prepared. The function of the other type of subunit is not known.

Recently we have described the purification of an aminopeptidase from *Bacillus stearothermophilus* with an apparent molecular weight of 400,000 (Zuber and Roncari, 1967; Roncari and Zuber, 1969; Moser *et al.*, 1970). This enzyme was designated aminopeptidase I as *B. stearothermophilus* also contains two low molecular weight aminopeptidases. The high molecular weight aminopeptidase I is thermostable up to 80° and has a specific quaternary structure. This enzyme could be split into subunits, which showed a molecular weight of 36,000 and appeared to be homogeneous on disc electrophoresis at alkaline pH in the presence of urea, as well as in the analytical ultracentrifuge (Moser *et al.*, 1970). Amino-terminal sequence analyses however showed two different peptide chains and we have therefore reinvestigated the subunit structure of this enzyme. These studies clearly show that aminopeptidase I consists of two different types of subunit which can combine in different ratios.

Experimental Section

Materials. B. stearothermophilus cells (strain NCIB 8924) were a gift from Ciba-Geigy AG, Basle, Switzerland. Cellogel electrophoresis strips were obtained from Chemetron, Milan, Italy. Leucine-*p*-nitroanilide was purchased from Serva, Heidelberg, Germany, and glycyl-L-leucyl-L-tyrosine from Fluka AG, Buchs, Switzerland. All other chemicals were highly purified commercial products.

Enzyme Assay. Aminopeptidase I activity was measured spectrophotometrically by following the hydrolysis of a 1 mM leucine-*p*-nitroanilide solution at 405 nm. The usual assay conditions were: 0.05 M imidazole hydrochloride buffer, containing 1 mM cobalt(II) chloride. The temperature was 55°; the pH was previously adjusted to 7.4 at room temperature. At a concentration of 1 mM leucine-*p*-nitroanilide does not saturate the enzyme. The Michaelis constant in imidazole hydrochloride buffer is 1.3 and 7 mM in Tris buffer. However at leucine-*p*-nitroanilide concentrations above 1 mM substrate inhibition becomes quite severe.

Enzyme Purification. Cells (500 g) were routinely suspended in 1.5 l. of 0.05 M Tris-HCl buffer (pH 7.2) (adjusted at room temperature) containing 1 mM cobalt(II) chloride (Tris–cobalt buffer). The cells were disrupted in a Manton–Gaulin press and centrifuged at 25,000*g* for 30 min. The supernatant was saved. Solid ammonium sulfate up to 47.5% saturation was added (295 g/l.). The pellet was discarded after centrifugation and the supernatant was brought to 75% saturation with solid ammonium sulfate (192 g/l.). The suspension was centrifuged and the supernatant discarded. The pellet was dissolved in 250 ml of Tris–cobalt buffer. The enzyme was then purified to a homogeneous state by Sephadex G-150 filtration, heat treatment, DEAE-Sephadex A-50 chromatography and preparative polyacrylamide gel electrophoresis

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