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Purification and Properties of the Wild Type and a Feedback-resistant Phosphoribosyladenosine Triphosphate

PYROPHOSPHATE PHOSPHORIBOSYLTRANSFERASE, THE FIRST ENZYME OF HISTIDINE BIOSYNTHESIS IN *SALMONELLA TYPHIMURIUM*

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SUMMARY

A purification procedure has been devised for phosphoribosyltransferase, the L-histidine-sensitive first enzyme in the pathway for histidine biosynthesis in *Salmonella typhimurium*. The procedure was applied to a wild type and a feedback-resistant strain. The enzymes from both strains appeared nearly homogeneous in the ultracentrifuge and upon polyacrylamide gel electrophoresis in urea and sodium dodecyl sulfate. The enzymes had similar sedimentation coefficients in the ultracentrifuge and similar mobilities on polyacrylamide gels containing sodium dodecyl sulfate. Tryptic peptide maps of the two enzymes could not be distinguished.

The wild type enzyme gave regular Michaelis-Menten kinetics but initial velocity analysis at a constant optimal magnesium to ATP ratio (2:1) gave nonparallel lines on double reciprocal plots. L-Histidine was an uncompetitive inhibitor with respect to phosphoribosyl pyrophosphate, while it was a noncompetitive inhibitor with respect to ATP. The curves for L-histidine and L-thiazolealanine inhibition were sigmoid in shape, and conversion to Hill plots gave straight lines with slopes of 1.6 and 1.8, respectively. Inhibition by both effectors was pH-dependent. The reverse reaction was also inhibited by L-histidine.

A difference spectrum of the wild type enzyme showed a striking increase in absorbance at 280 mμ upon the addition of L-histidine, whereas that of the feedback-resistant enzyme remained constant following the addition of L-histidine.

N-1-(5′-Phosphoribosyl) adenosine triphosphate:pyrophosphate phosphoribosyltransferase (EC 2.4.2) catalyzes the initial committed reaction in the L-histidine biosynthetic pathway of *Salmonella typhimurium* (1).

$$\text{ATP} + \text{5-phosphoribosyl 1-pyrophosphate} \xrightleftharpoons{Mg^{++}} N\text{-1-(5′-phosphoribosyl)-ATP} + \text{pyrophosphate}$$

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The gene (*hisG*) that codes for phosphoribosyltransferase activity is located in a cluster of nine genes on the *S. typhimurium* chromosome. These genes specify the structures of all of the enzymes for histidine biosynthesis. The genes are clustered in the order *operator G-D-C-B-H-A-F-I-E*. Regulated as a unit, they constitute the histidine operon (see review by Ames *et al.* (2)). The phosphoribosyltransferase is of interest because it is subject to feedback inhibition by the end product of the pathway, L-histidine (1). Martin (3) has conducted a detailed study of the wild type phosphoribosyltransferase with special emphasis on the feedback inhibition mechanism, with partially purified preparations of the enzyme. The wild type enzyme has recently been purified (4) to near homogeneity.

Sheppard (5) has isolated a series of mutants that are resistant to the histidine analogue, thiazolealanine, and have lesions lying in the *G* gene. Each of these thiazolealanine-resistant mutants contains a feedback-resistant phosphoribosyltransferase. In order to elucidate any physical or chemical differences between the wild type and feedback-resistant enzymes, the two proteins have been purified to near homogeneity with a new purification scheme. This purification procedure, the kinetic and feedback properties of the purified wild type enzyme, and a comparison between the two proteins form the subject of this report.

MATERIALS AND METHODS

Chemicals

Dimagnesium PP-ribose-P[1] was obtained from P-L Biochemicals. Solutions of 10 mM PP-ribose-P concentration were made up in 20 mM sodium EDTA, pH 7.0, neutralized to pH 7.0 with Tris base, and stored frozen. The PP-ribose-P concentrations stated are based on the results of an orotidine 5′-phosphate pyrophosphorylase assay for PP-ribose-P done by the manufacturer. For kinetic studies in which the Mg:ATP ratio remained constant, the PP-ribose-P solutions did not contain EDTA. Disodium ATP, tetrasodium pyrophosphate, and yeast inorganic

[1] The abbreviations used are: PP-ribose-P, 5-phosphoribosyl α-1-pyrophosphate; PR-AMP and PR-ATP, *N*-1-(5′-phosphoribosyl) adenosine mono- and triphosphate; BBM III, *N*-(5′-phospho-D-1′-ribulosylformimino)-5-amino-1)5″-phosphoribosyl)-4-imidazolecarboxamide; SDS, sodium dodecyl sulfate.

pyrophosphatase (crystallized twice) were obtained from Sigma. Mallinckrodt urea, analytical grade, was used without further purification. Trypsin, twice crystallized, treated with diisopropyl fluorophosphate (6), was supplied by Worthington. Iodoacetic acid (twice recrystallized from petroleum ether), special enzyme grade ammonium sulfate, and recrystallized bovine insulin were purchased from Mann. Cyclo Chemicals supplied DL-2-thiazolealanine.

Bacterial Strains

The wild type phosphoribosyltransferase was isolated from strain *hisT1504 hisC146*,[2] which contains a histidine-constitutive mutation, *hisT1504* (7), and a frameshift mutation, *hisC146* (8). This strain was constructed by transducing *hisC146* into strain *hisT1504 hisOGDCBH2253* (9). The phage mutant L4 of P22 (10) was used in the transduction, and auxotrophic recombinants capable of growth on histidinol (D gene function) were selected. A nonlysogenic recombinant was picked and used as a source for the wild type enzyme.[3] The feedback-resistant phosphoribosyltransferase was isolated from strain *hisG1109 hisIF135* (5).

Growth of Bacterial Strains

Cells were cultured in a 300-liter fermenter at 37° and harvested in the late log phase of growth by centrifugation.[4]

Mutant *hisT1504 hisC146* was grown in Medium E of Vogel and Bonner (11) containing 0.1 mM L-histidine. Use of the sodium citrate[5] in Medium E as the sole carbon source resulted in a 30-fold derepression of the phosphoribosyltransferase in constitutive cells as compared to wild type strain LT2 cells grown under the same conditions. Cells were grown in the fermenter from a starting inoculum of 500 cc. With forced aeration, mutant *hisT1504 hisC146* grew with a generation time of approximately 80 min in the fermenter. The yield of late log phase cells after 16 hours of growth was 400 to 500 g (wet weight) of cells.

The feedback-resistant strain, *hisG1109 hisIF135*, was grown in the fermenter on Medium E (11) containing 0.5% glucose, 0.06 mM histidinol, 0.0075 mM L-histidine, and 0.4 mM adenine. These conditions result in up to a 25-fold derepression of the histidine biosynthetic enzymes as compared to organisms grown on excess histidine (12). The fermenter yield of cells from a 1-liter inoculum after 16 hours of growth was 900 g (wet weight) of cells.

Enzyme Assays

Two assays for the forward reaction were used in this study.

Coupled G-70 Assay—The coupled assay is based on the conversion of the product of the forward reaction, PR-ATP, to BBM III (1). BBM III has an extinction coefficient of 8.0×10^3 M^{-1} cm^{-1} at 290 mμ (12). An extract of strain *hisG70* provided an excess of the enzymes required to convert PR-ATP to BBM III. This assay can be used for assaying any strain regardless of whether or not the strain has the enzymes for conversion of PR-ATP to BBM III (1). The reaction mixture, similar to that previously reported (4), consisted of 30 μmoles of Tris-HCl buffer (pH 8.5), 3 μmoles of $MgCl_2$, 45 μmoles of KCl, 1.5 μmoles of ATP, 0.15 μmole of PP-ribose-P, 5 μl of an extract of strain *hisG70* that had been passed through a Sephadex G-50 column (13), and enzyme in a final volume of 0.3 ml. To initiate the reaction, PP-ribose-P was added and the initial change in absorbance at 290 mμ was followed on a Beckman spectrophotometer equipped with a Gilford multiple sample absorbance recorder. When enzyme, either of the substrates, or magnesium chloride was omitted from the reaction mixture, no appreciable change in absorbance occurred.

One unit of activity is defined as an initial change in absorbance at 290 mμ of 0.10 per 5 min. Based upon the extinction coefficient of BBM III (12), 1 unit of activity corresponds to the formation of 0.75 nmole of BBM III per min. The assay is linear with time and enzyme concentration to an initial change in absorbance of 0.150 per 5 min at 290 mμ.

Pyrophosphatase Assay—The assay measures the formation of PR-ATP at 290 mμ and has been previously described (4). This assay was used in studies of the purified enzyme. The routine assay gave identical reaction rates regardless of whether or not the solutions of PP-ribose-P contained EDTA. As previously defined (4), 1 unit of activity is an initial change in absorbance of 0.10 per 5 min and corresponds to the formation of 1.67 nmoles of PR-ATP per min. The units of enzyme activity obtained from this assay can be converted to units of enzyme activity for the coupled *G-70* assay by multiplying by 2.9.

Assay of Reverse Reaction—The assay of the reverse reaction depends on the determination of the rate of decrease in absorbance at 290 mμ of PR-ATP. Neither ATP nor PR-ATP absorbs appreciably at 290 mμ (1). The reaction mixture contained 10 μmoles of Tris-HCl buffer (pH 8.5), 0.3 μmole of tetrasodium pyrophosphate, 6.8 nmoles of PR-ATP, 0.5 μmole of $MgCl_2$, and enzyme in a total volume of 0.3 ml. PR-ATP was added to start the reaction, and the initial change in absorbance at 290 mμ was followed. The assay was linear with time and enzyme concentration to an initial change in absorbance of 0.02 per 4 min. One unit of activity is defined as an initial change in absorbance of 0.1 per 4 min. This corresponds to the disappearance of 2.08 nmoles of PR-ATP per min. The enzymatic synthesis of PR-ATP is described below.

Polyacrylamide Gel Electrophoresis

Electrophoresis of the enzyme in polyacrylamide gels containing urea was performed according to the method of Reisfeld and Small (14). Gels were stained for proteins with a 0.25% solution of Amido black (Allied Chemicals) in 7% acetic acid for 1 hour and were destained with 7% acetic acid.

The subunit molecular weight of the wild type and feedback-resistant enzymes was determined in 7.5% polyacrylamide gels containing SDS as described by Shapiro, Viñuela, and Maizel (15).

[2] The nomenclature is that of Dr. P. E. Hartman. The letters designate the gene(s) affected by a mutation and are followed by a mutation number. Mutations in the *T* gene, which lie outside of the histidine operon, are constitutive for the histidine biosynthetic enzymes (7). The *1100* series of mutants isolated by Sheppard (5) contain feedback-resistant phosphoribosyltransferase enzyme.

[3] Strain *hisC146* contains a frameshift mutation which is a polar mutation (8). It was necessary to construct a strain carrying a polar mutation because Voll (personal communication) has shown that histidine-constitutive nonpolar strains produce an unidentified substance which retards the flow rates of column chromatography. Repeated attempts to construct an equivalent strain containing a polar mutation and a histidine-constitutive mutation for the feedback-resistant mutant failed.

[4] The authors are indebted to D. Rogerson, D. Johnson, and J. Hicks who carried out the fermenter runs.

[5] T. Kłopotowski, personal communication.

Absorption Spectra

Difference absorption spectra were measured in a Cary model 15 recording spectrophotometer with cuvettes with a 1-cm light path. Enzyme was prepared for these studies by overnight dialysis against 2 liters of the buffer described by Voll, Appella, and Martin (4) at pH 8.6 and containing 0.01 M β-mercaptoethanol. The temperature was 25°.

Enzymatic Synthesis of PR-ATP and PR-AMP

PR-ATP and PR-AMP were generated in a cell-free extract of mutant *hisT1504 hisHAFIE2327* (9) by the method of Ames, Martin, and Garry (1) as modified by Smith and Ames (16). The PR-AMP and PR-ATP were separated from the crude reaction mixture by chromatography on DEAE-cellulose as described by Smith and Ames (16). The PR-ATP fraction was rechromatographed once. The purified PR-ATP had an $A_{260}:A_{290}$ ratio of 5.38 at pH 8.5, the PR-AMP had an $A_{260}:A_{290}$ ratio of 10 at pH 8.5 (16).

Ultracentrifugation

Sedimentation velocity studies were done in a Beckman model E analytical ultracentrifuge equipped with an ultraviolet scanner. Protein was prepared for these studies by dialysis against 2 liters of buffer containing 0.01 M Tris (pH 8.5), 0.1 M NaCl, and 0.01 M β-mercaptoethanol. These studies were carried out at a speed of 60,000 rpm.

Enzyme Reduction and Carboxymethylation

Reduction and carboxymethylation of the enzyme were carried out by the method of Craven, Steers, and Anfinsen (17).

Tryptic Digestion and Peptide Mapping

Lyophylized carboxymethylated enzyme was dissolved to 1 mg per ml in 0.2 N ammonium bicarbonate. Three aliquots of trypsin were added to a final enzyme to substrate ratio of 1:40 (w/w), and the digestion mixture was incubated at 37° for 5 hours. Following lyophilization of the tryptic digest, peptide maps were prepared on Whatman No. 3MM chromatography paper by the method of Katz, Dreyer, and Anfinsen (18). The chromatography in 1-butanol-acetic acid-water (4:1:5) was performed without prior equilibration of the paper. Electrophoresis in pH 3.6 pyridine acetate buffer was carried out for 110 min at 2000 volts. Peptides were stained with the cadmium-ninhydrin reagent of Dreyer and Bynum (19).

Protein Determination

Protein concentration, during the purification procedure, was estimated by the method of Lowry *et al.* (20) with insulin as a standard. The protein determination of fractions prior to the Sephadex G-150 chromatography step was performed on material that had been passed through a Sephadex G-50 column (13). The nitrogen content of the purified enzyme was assayed by the micro-Kjeldahl technique. Quadruplicate analyses were conducted by the method of Ma and Zuazaga (21).[6]

[6] Nitrogen determinations were generously performed by Dr. W. C. Alford of the Microanalytical Chemistry Laboratory, National Institute of Arthritis and Metabolic Diseases, National Institutes of Health.

RESULTS

Purification of Wild Type Phosphoribosyltransferase

Buffer—The standard buffer consisted of 0.01 M Tris-HCl (pH 7.5), 0.1 M NaCl, 0.4 mM histidine, and 0.5 mM EDTA as previously described (4); however, β-mercaptoethanol was replaced by 1 mM dithiothreitol. Buffer A differs from the standard buffer by containing 0.1 M Tris-HCl, pH 7.5.

Preparation of Extracts—All procedures were performed at 4° unless otherwise stated. In a typical purification, 1000 g, wet weight, of cells were suspended in a minimal volume of Buffer A and homogenized in a Waring Blendor at half-minimal speed for 5 min. Following homogenization, Buffer A was added to a total volume of 2 liters. The cells were disrupted by two passages through a Branson sonifier, equipped with a 0.5-inch probe and a continuous flow attachment, at a current of 5 amp. The cell debris was removed by centrifugation at 27,000 $\times$ *g* for 70 min in a Servall RC-2B centrifuge.

Heat Step—The pH of the supernatant fraction was increased to 7.0 with 1 M Tris base. This fraction was then distributed in 500-ml fractions into 1-liter Erlenmeyer flasks. A temperature of 61° was attained in 5 min by immersion of the flask, with vigorous agitation, into a water bath at 70°. The temperature of the contents of the flask was maintained at 61° by agitation in a 61° water bath for 8 min. Rapid cooling to 5° was achieved by immersion of the flask in an ice-salt bath. A supernatant fraction was obtained after centrifugation at 27,000 $\times$ *g* for 50 min. The heat step generally gave a 3-fold purification with 75 to 85% recovery of the input activity.

Ammonium Sulfate Fractionation—Ammonium sulfate, 22.6 g/100 ml of supernatant fraction, was added slowly with stirring (4). The pH was maintained at 7.0 by addition of 1 M Tris base. The ammonium sulfate suspension was stirred for 30 min, and then was centrifuged at 27,000 $\times$ *g* for 20 min. Twelve grams of ammonium sulfate per 100 ml of original volume were added to the supernatant solution as described above. Following centrifugation at 27,000 $\times$ *g* for 20 min, the precipitate was redissolved in 40 to 60 ml of Buffer A. This material was placed in a dialysis bag that had been boiled for 5 min in 10^{-4} M EDTA. Dialysis against 4 liters of Buffer A at pH 8.0 was performed for 3 hours.

Sephadex G-150 Chromatography—The dialyzed ammonium sulfate fraction was applied to a column of Sephadex G-150, 229 $\times$ 5 cm, 4.3 liters, previously equilibrated with standard buffer. The enzyme was eluted with this buffer at a rate of 40 to 50 ml per hour. After the passage of approximately 1800 ml of buffer, enzymatic activity appeared just behind the excluded material. Fractions of 20 ml were collected, and those containing enzyme with the highest specific activity were combined.

DEAE-Sephadex A-50 Chromatography—The volume of the combined fractions from the Sephadex G-150 step was doubled with cold-distilled water. To this solution, 2.8 mmoles of β-mercaptoethanol per 100 ml were added and the pH was increased to 8.6 with 1 M Tris base. DEAE-Sephadex A-50, equilibrated with standard buffer at pH 8.6 and 0.05 M NaCl, was used to fill a column, 36 $\times$ 2.5 cm, of 150-ml bed volume. Following the application of the enzyme solution to the column, 1 to 2 column volumes of the equilibration buffer were passed through the column. The protein was eluted with a 1400-ml continuous linear gradient of NaCl (0.05 to 0.5 M) in the equilibration buffer. The flow rate of the column was 0.5 ml per min, and fractions of

TABLE I

Modified purification of N-1-(5'-phosphoribosyl)-ATP: pyrophosphate phosphoribosyltransferase of Salmonella typhimurium

Procedure	Volume	Activity	Protein[a]	Specific activity	Yield	Inhibition by 9.9×10^{-6} M histidine
	ml	*units*	*mg*	*units/mg*	%	%
Crude extract	1,638	1.11×10^6	11,000	101		83
Heat step	1,520	9.71×10^5	2,584	375	87	85
Ammonium sulfate	32	6.59×10^5	640	1,030	59	89
Sephadex G-150	158	4.68×10^5	75	6,240	42	79
DEAE-Sephadex	50	3.95×10^5	45	8,780	36	80

[a] Determined by the method of Lowry *et al.* (20).

6 ml were collected. Fractions containing enzyme of the highest specific activity were combined.

Concentration and Storage of Enzyme—The combined fractions were concentrated 3-fold in an Amicon ultrafiltration cell equipped with a UM-1 filter.[7] The concentrated enzyme solution was then made 3 M in NaCl[8] and 0.01 M in dithiothreitol and stored at −20°. The specific activity of the purified enzyme stored as indicated decreased 50% over a period of 2 weeks.

A summary of this procedure is presented in Table I. Similar results have been obtained on three other occasions. The final concentrated enzyme has a specific activity of 8800 units per mg of protein based on the coupled *G-70* assay and a protein determination by the method of Lowry *et al.* (20). A specific activity of 3000 units per mg of protein was obtained when the enzyme was assayed by the pyrophosphatase assay. Quadruplicate nitrogen analyses were performed on the purified enzyme following extensive dialysis against distilled water. The nitrogen content and the percentage weight nitrogen in the enzyme (18%) were used to calculate a protein concentration. On the basis of this value and with the coupled *G-70* assay, the specific activity of the enzyme was 6600 units per mg of protein.

Purity of Wild Type Phosphoribosyltransferase

The purified enzyme was judged to be nearly homogeneous on the basis of ultracentrifugation analyses, electrophoresis on polyacrylamide gels in urea, and electrophoresis on polyacrylamide gels in SDS.

Sedimentation Velocity—At a protein concentration of 1.4 mg per ml, a single symmetrical peak with an $s_{20,w}$ of 8.94 S was observed. This value is in good agreement with the value of 8.83 S reported previously (4).

Disc Gel Electrophoresis—Electrophoresis of 50 μg of protein on polyacrylamide gels containing urea (14) revealed one dark staining band and two minor bands (Fig. 1). At 100 μg of protein two additional faint bands appeared. Although these minor bands may represent contaminants, the possibility that they are isozymes or aggregates has not been ruled out. On electrophoresis in polyacrylamide gels without urea multiple bands appeared.

[7] Amicon Corporation, Lexington, Massachusetts. The UM-1 membrane retains material with a molecular weight of 10,000. Nitrogen pressure was maintained at 40 to 50 p.s.i.

[8] Dr. J. Feretti, personal communication.

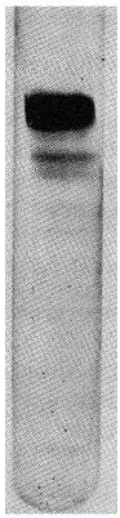


FIG. 1. Polyacrylamide gel electrophoresis in urea of purified wild type phosphoribosyltransferase. Acrylamide gel electrophoresis of 50 μg of enzyme was conducted as described by Reisfeld and Small (14).

Electrophoresis of 275 μg of protein in polyacrylamide gels containing SDS showed one major band and one minor band (see below).

Purification of Feedback-resistant Enzyme

Phosphoribosyltransferase was also purified from the mutant, *hisG1109 hisIF135* (5), in which the feedback inhibition site of the enzyme is insensitive to histidine. The purification procedure was identical with that described for the wild type enzyme except for the following differences: (*a*) the histidine concentration in the standard buffer was increased to 0.01 M, and (*b*) the cells were disrupted by two passages through a Gaulin Laboratory homogenizer[9] at 10,000 p.s.i. Preliminary experiments had shown that 0.01 M histidine was required to stabilize the mutant enzyme for the heat step. The mutant enzyme behaved in a similar manner to the wild type enzyme throughout the purification procedure. The specific activity of the purified feedback-resistant enzyme based on the coupled *G-70* assay and the protein method of Lowry *et al.* (20) was 6,200 units per mg of protein. A 78-fold purification with 18% recovery of the starting activity was obtained. The purified preparation showed no inhibition by 6.6×10^{-3} M histidine.

Purity of Feedback-resistant Phosphoribosyltransferase Enzyme—Electrophoresis of 50 μg of purified feedback-resistant enzyme in polyacrylamide gels containing urea showed a pattern identical with that observed with the wild type enzyme. One major and one minor band were noted on SDS gel electrophoresis. During sedimentation velocity studies at a protein concentration of 1.4 mg per ml, a single symmetrical peak with an $s_{20,w}$ of 9.16 S was observed. Insufficient analyses were carried out to determine whether or not this value is significantly different from 8.94 S.

Properties of Purified Wild Type Phosphoribosyltransferase

Kinetics of Forward Reaction—A constant magnesium ($MgCl_2$) to ATP ratio of 2:1 (except Fig. 4, *A* and *B*) was chosen for the kinetic analysis since this ratio gave optimal reaction rates.

[9] Monton-Gaulin Manufacturing Corporation, Everett, Massachusetts.

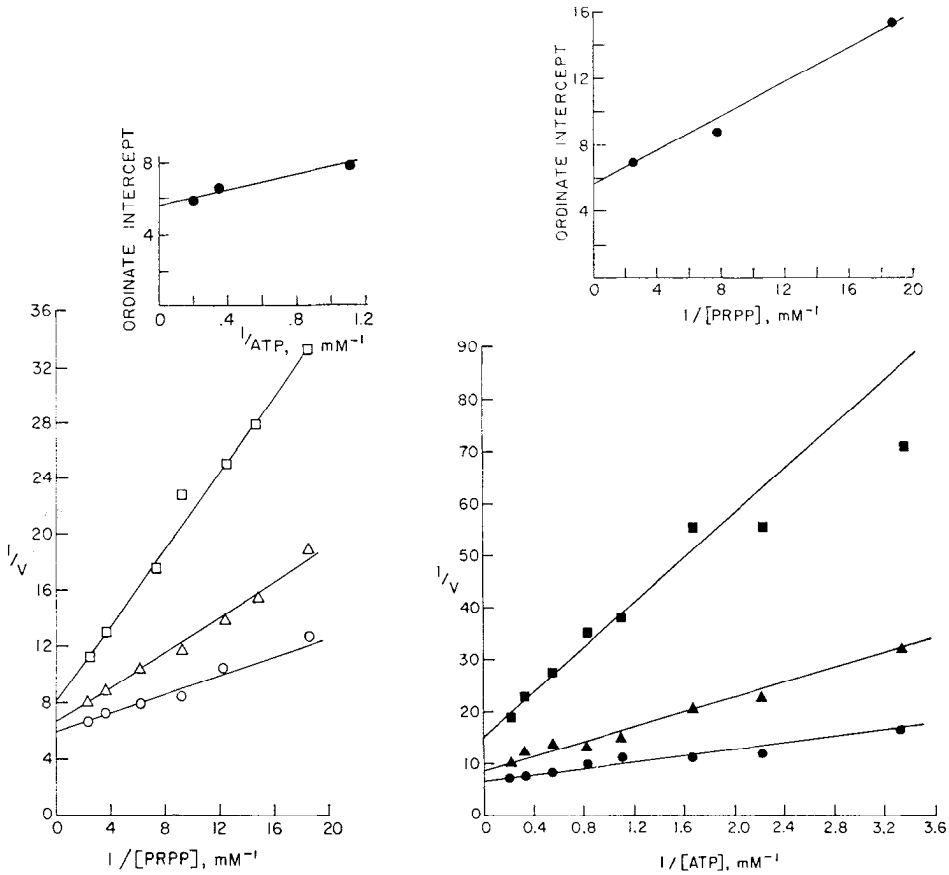


FIG. 2 (*left*). Double reciprocal plot of initial velocity against PP-ribose-P (*PRPP*) concentration. The initial velocity is expressed as the reciprocal ($1/V$) of the initial change in absorbance at 290 mμ per 5 min. The magnesium to ATP ratio was constant at 2:1 ○——○, 4.48 mM ATP; △——△, 2.99 mM ATP; □——□, 0.87 mM ATP. *Inset*, secondary plot of the ordinate intercepts against 1/[fixed substrate, ATP] of the data in the main figure.

FIG. 3 (*right*). Double reciprocal plot of initial velocity against ATP concentration. The initial velocity is expressed as the reciprocal ($1/V$) of the initial change in absorbance at 290 mμ per 5 min. The magnesium to ATP ratio was constant at 2:1. ●——●, 0.400 mM PP-ribose-P (*PRPP*): ▲——▲, 0.133 mM PP-ribose-P; ■——■, 0.0534 mM PP-ribose-P. *Inset*, secondary plot of the ordinate intercepts against 1/[fixed substrate, PP-ribose-P] of the data in the main figure.

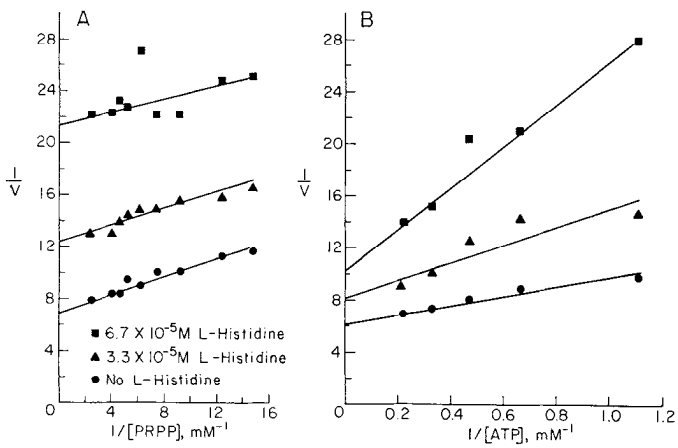


FIG. 4. Reciprocal initial velocity plotted against reciprocal substrate concentrations in the presence and absence of L-histidine. A constant magnesium concentration of 10 mM was maintained. The initial velocity is expressed as the reciprocal ($1/V$) of the initial change in absorbance at 290 mμ/5 min. *A*, reciprocal velocity against reciprocal PP-ribose-P (*PRPP*) concentration at various L-histidine concentrations. ●——●, no L-histidine; ▲——▲, 3.3×10^{-5} M L-histidine; ■——■, 6.7×10^{-5} M L-histidine. *B*, reciprocal velocity against reciprocal ATP concentration at various L-histidine concentrations. ○——○, no L-histidine; △——△, 3.3×10^{-5} M L-histidine; ■——■, 6.7×10^{-5} M L-histidine. *PRPP*, PP-ribose-P.

The apparent K_m for PP-ribose-P (with the standard ATP concentration) was obtained from a Lineweaver-Burk plot (22) and is 5.6×10^{-5} M (Fig. 2). The apparent K_m for ATP (with the standard PP-ribose-P concentration) is 4.3×10^{-4} M (Fig. 3). These Lineweaver-Burk plots do not deviate from linearity. In order to derive concentration-independent Michaelis constants, secondary plots of the ordinate intercepts against 1/[fixed substrate] were drawn (Figs. 2 and 3, *insets*). The concentration-independent Michaelis constant for ATP is 4.2×10^{-4} M (Fig. 2, *inset*), while the concentration-independent Michaelis constant for PP-ribose-P is 1.0×10^{-4} M (Fig. 3, *inset*). When the magnesium concentration was maintained at 10 mM, the apparent K_m for PP-ribose-P (standard ATP concentration) was 5.1×10^{-5} M (Fig. 4*A*) and that for ATP (standard PP-ribose-P concentration) was 4.8×10^{-4} M (Fig. 4*B*).

Double reciprocal plots of initial velocity against PP-ribose-P concentration at different fixed concentrations of ATP resulted in a family of lines that intersect to the left of the ordinate above the abscissa (Fig. 2). The same pattern was seen when ATP

TABLE II

Reversibility of phosphoribosyltransferase reaction

The complete system was as described under "Materials and Methods"; 0.026 mM PR-AMP and 0.5 mM L-histidine were added as indicated.

Reaction mixture	Enzyme activity
	units/ml
Complete	324
Minus phosphoribosyltransferase	0
Minus PR-ATP	0
Minus PP_i	0
Minus Mg^{++}	0
Minus PR-ATP, plus PR-AMP	0
Plus L-histidine	137

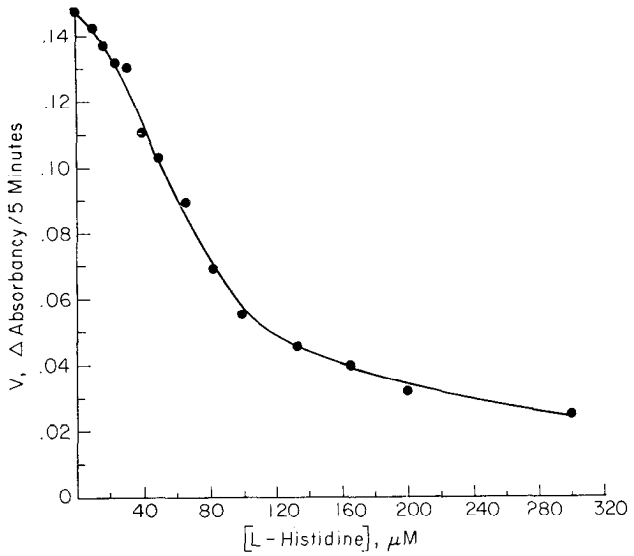


FIG. 5. L-Histidine inhibition of the forward reaction. Assays were conducted as described under "Materials and Methods" except that L-histidine was added at the indicated micromolar concentrations.

concentration was varied as a function of several fixed concentrations of PP-ribose-P (Fig. 3). The nonparallel pattern of the initial velocity plots suggests that under these conditions the predominant mechanism of the forward reaction is one involving a ternary complex (23).

Reversibility of Reaction—The reversibility of the phosphoribosyltransferase reaction, *i.e.* formation of PP-ribose-P and ATP from PR-ATP and PP_i in the presence of Mg^{++}, has been shown (1). Table II depicts the absolute requirement of this reaction for enzyme, both substrates, and magnesium. Substitution of PR-AMP for PR-ATP yielded no reaction. Interestingly, the reverse reaction was inhibited by histidine; 50% inhibition was observed at 4×10^{-4} M histidine.

Feedback Inhibition of Purified Phosphoribosyltransferase

L-Histidine Inhibition—Table I illustrates that the L-histidine sensitivity of the enzyme remained approximately constant throughout the purification. The concentration of L-histidine required for 50% inhibition of activity was 7×10^{-5} M. In another preparation, 8×10^{-5} M L-histidine inhibited activity by

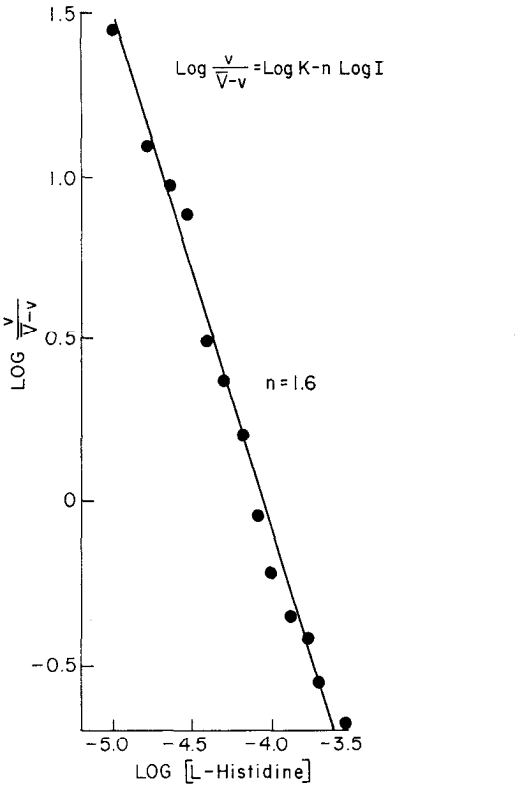
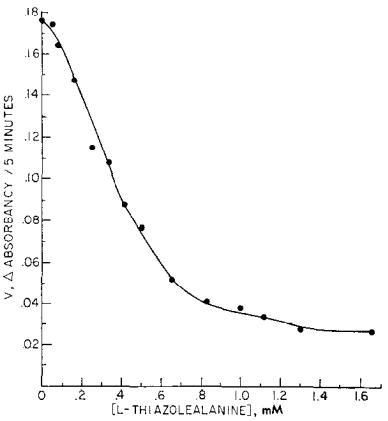


FIG. 6. Modified Hill plot (25, 26) of Fig. 5. V was the initial velocity in the absence of L-histidine, v was the initial velocity in the presence of L-histidine, I was the concentration of L-histidine, and n was the slope of the line.



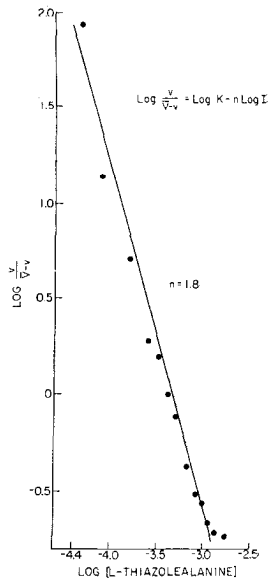


FIG. 7 (*left*). Thiazolealanine inhibition (with respect to the L isomer) of the forward reaction. Assays were conducted as described under "Materials and Methods" except that thiazolealanine was added at the indicated millimolar concentrations for the L isomer.

FIG. 8 (*right*). Modified Hill plot (25, 26) of Fig. 7. The *symbols* used in the equation are described in the legend of Fig. 6.

50%. Previously reported preparations of the purified enzyme were inhibited 50% by 6×10^{-5} M and 9×10^{-5} M L-histidine (4).

When the PP-ribose-P concentration was varied (Fig. 4*A*), L-histidine was an uncompetitive inhibitor altering both V_{max}

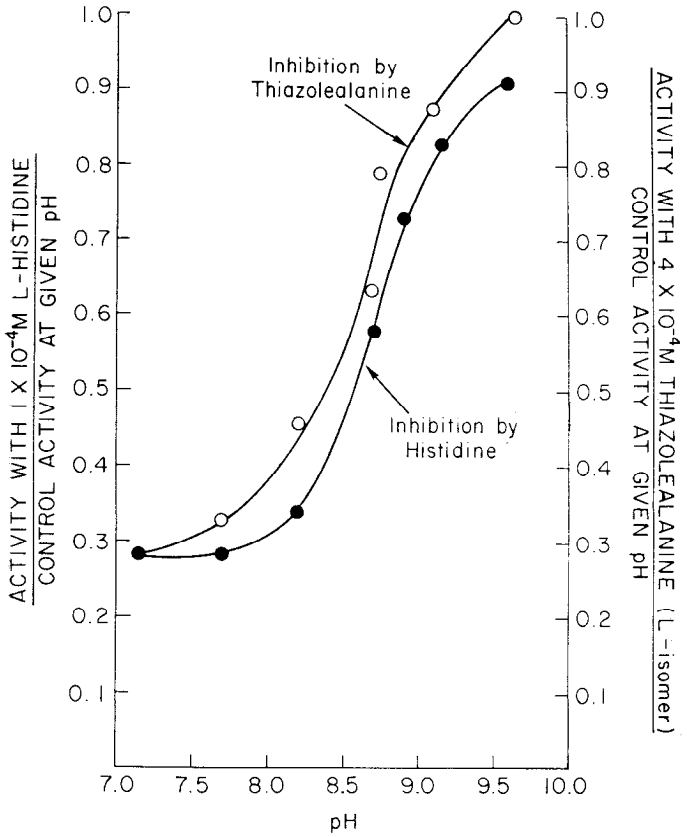


FIG. 9 Dependence of L-histidine and thiazolealanine inhibition on pH. The pH of the assay mixture was measured at the end of each assay.

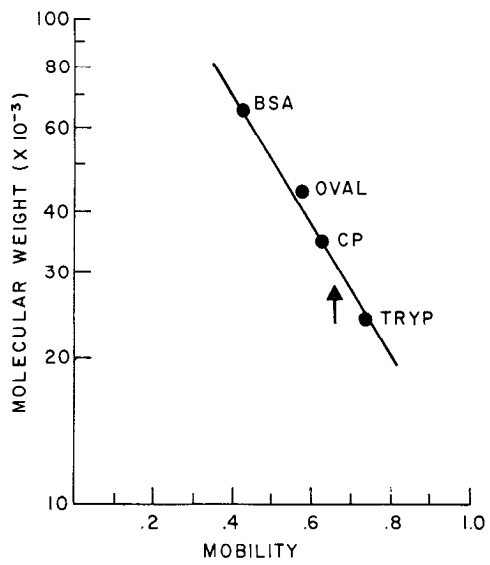


FIG. 10. Determination of the subunit molecular weight of the wild type and feedback-resistant phosphoribosyltransferases. The *arrows* indicate the mobility of the phosphoribosyltransferases. *BSA*, bovine serum albumin, molecular weight 65,400 (31); *Oval*, ovalbumin, molecular weight 44,000 (31); *CP*, carboxypeptidase A, molecular weight 34,600 (32); *TRYP*, trypsin, molecular weight 24,500 (33). The SDS polyacrylamide gel analysis was performed by the method of Shapiro *et al.* (15). Each gel was loaded with 275 μg of wild type enzyme or 198 μg of feedback-resistant enzyme.

and K_m (24). Inhibition by L-histidine was noncompetitive (24) when the ATP concentration was varied (Fig. 4*B*).

Fig. 5 illustrates that a sigmoid curve was obtained when the activity of the forward reaction was followed as a function of L-histidine concentration. A secondary plot of these data (Fig. 6), with the modified Hill equation (27), yielded a linear plot with a slope (n) of 1.6. The fact that the slope is greater than 1 and less than 2 suggests the presence of at least two interacting binding sites on the enzyme for histidine. The value of n varied with the age of the enzyme and approached 1 in older preparations.

Thiazolealanine Inhibition—Moyed and Friedman (28) and Moyed (29) initially showed that thiazolealanine, a histidine analogue, mimics L-histidine in its inhibition of the BBM III-synthesizing system of *Escherichia coli*. Ames *et al.* (1) and Martin (3) subsequently demonstrated that the inhibition observed was due to inhibition of the phosphoribosyltransferase.

When the rate of the forward reaction was followed as a function of thiazolealanine concentration (with respect to the L isomer), a sigmoid curve was observed (Fig. 7). Fifty per cent inhibition occurred at 4×10^{-4} M thiazolealanine. Use of the modified Hill equation yielded a straight line with a slope (n) of 1.8 (Fig. 8). This value of n is also consistent with the presence of at least two interacting binding sites on the enzyme for thiazolealanine. The value of n was 1 in older preparations of the enzyme.

L-Histidine and Thiazolealanine Inhibition as Function of pH—Fig. 9 shows that feedback inhibition by L-histidine and thiazolealanine (with respect to the L isomer) is pH-dependent with maximal inhibition by each effector occurring in the physiological pH range. The curve for inhibition by each molecule is similar to that expected for the titration curve of a single group with an approximate pK of 8.7. In a similar experiment with partially purified enzyme, Martin (3) reported a value of 9.2. The pK of the α-amino group of L-histidine is 9.2 (30). The difference between the pK obtained in this experiment and the pK of L-histidine suggests that the approximate pK of 8.7 represents the pK of the interaction of L-histidine with a group (groups) on the enzyme.

Comparison of Wild Type and Feedback-resistant Phosphoribosyltransferases

Subunit Molecular Weight in SDS Gels—The mobility of both enzymes in polyacrylamide gels containing SDS was measured with proteins of known molecular weights as standards (15). The mobilities of the wild type and feedback-resistant enzymes were identical, 0.66 (Fig. 10). These mobilities correspond to an approximate molecular weight of 34,000 for the subunits of the wild type and feedback-resistant enzymes. A minor band of lower mobility was occasionally observed in both preparations.

Tryptic Peptide Maps—Peptide maps of the wild type enzyme were reproducible and revealed 34 to 36 ninhydrin-positive spots plus some core material at the origin. The peptide map of the feedback-resistant enzyme was indistinguishable from that of the wild type enzyme.

Sedimentation Velocity Studies—As reported above, the wild type enzyme had an $s_{20,w}$ of 8.94, while the feedback-resistant enzyme had an $s_{20,w}$ of 9.16. These values agree to within 3%.

Difference Spectra—A striking change was observed in the difference spectrum at 280 mμ of the wild type enzyme upon the addition of L-histidine (Fig. 11). The absorbance at 280

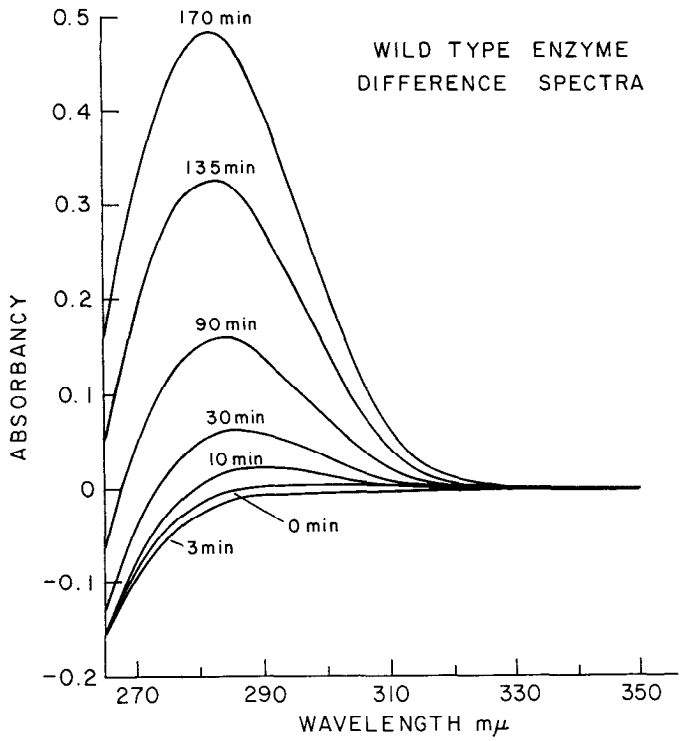


FIG. 11. Difference spectra of the purified wild type phosphoribosyltransferase. Both cuvettes contained 1 μmole of magnesium chloride and 0.4 mg of enzyme in 1 ml of a pH 8.6 buffer 0.01 M in β-mercaptoethanol (4). Sequential difference spectra remained constant after the addition of each component until the sample cuvette was made 0.01 M in L-histidine. Upon the addition of L-histidine a series of difference spectra were taken at the times indicated in the figure.

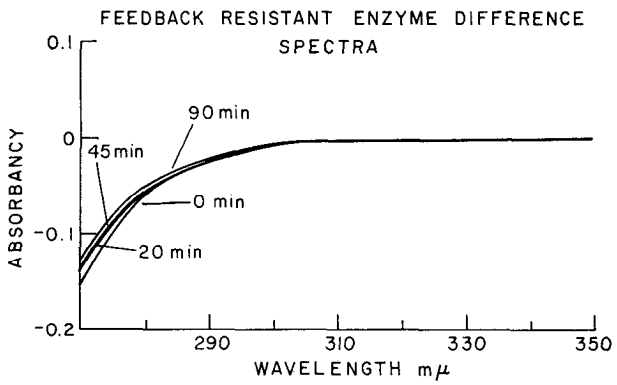


FIG. 12. Difference spectra of the purified feedback-resistant phosphoribosyltransferase. Both cuvettes contained 1 μmole of magnesium chloride and 0.3 mg of enzyme in 1 ml of 0.01 M β-mercaptoethanol, pH 8.6, buffer described by Voll *et al.* (4). Sequential difference spectra remained constant following the addition of each component. When the sample cuvette was made 0.01 M in L-histidine, a series of difference spectra was taken at the times indicated in the figure.

mμ continued to increase at 170 min after the addition of L-histidine to one of the cuvettes. This increase in absorbance at 280 mμ seems too great to be consistent with a conformational change in which buried aromatic amino acid residues are being exposed. However, no precipitate was observed in the cuvettes and the spectra do not appear to be consistent with the formation of a precipitate (absorption is not proportional to $1/\lambda^4$) although anomalous absorption due to precipitation cannot be excluded. The difference spectrum of the feedback-resistant enzyme remained constant (Fig. 12) following the addition of histidine. Whatever may be the basis for the anomalous absorption of the wild type enzyme upon the addition of histidine, these data suggest that the interaction of L-histidine with the enzyme leads to a conformational change which may be necessary for feedback inhibition of the enzyme.

DISCUSSION

The purified phosphoribosyltransferase was used to study the kinetic properties of the forward reaction. Measurements of velocity as a function of substrate concentration showed normal Michaelis-Menten kinetics for PP-ribose-P and ATP even in the presence of histidine. These results suggest that neither PP-ribose-P nor ATP undergoes homotropic interactions.

Initial velocity analysis, under optimal assay conditions, resulted in a family of intersecting lines on double reciprocal plots for both PP-ribose-P and ATP. Such kinetic patterns imply that under these conditions the predominant reaction sequence for PR-ATP synthesis is one involving a ternary complex of PP-ribose-P, ATP, and enzyme (23). In contrast to these results, it was earlier shown (3) that the partially purified enzyme catalyzes an exchange between $^{32}PP_i$ and PP-ribose-P in the absence of ATP and between ^{14}C-ATP and PR-ATP in the absence of PP-ribose-P. Recently Bell and Koshland (34), using purified phosphoribosyltransferase obtained by a modification of the procedure described in this paper, have isolated a phosphoribosyl-enzyme intermediate. The existence of a covalent enzyme-substrate complex suggests that under other circumstances PR-ATP synthesis may proceed via a "ping-pong" mechanism in which release of the first product precedes addition of the second substrate. The factors which cause the predominance of one reaction sequence over an alternative one are not known.

A general model (25) proposed to explain the properties of regulatory enzymes distinguished two types of regulatory systems (K and V) on the basis of kinetic properties. The kinetic properties of the phosphoribosyltransferase are closest to those of a negative V system. Neither substrate exhibits homotropic interactions, while the inhibitors, histidine and its analogue, thiazolealanine, both exhibit homotropic interactions. Alternatively, the kinetic properties of the enzyme could be accounted for by other models such as the one described by Koshland, Nemethy, and Filmer (35).

A guiding interest in this study has been the mechanism of feedback inhibition by histidine. Histidine was observed to be an uncompetitive inhibitor of the wild type phosphoribosyltransferase with respect to PP-ribose-P and gave noncompetitive inhibition with respect to ATP. It therefore seems unlikely that histidine inhibits by merely binding to the active site.

The sigmoid curves for histidine and thiazolealanine inhibition are consistent with the cooperative binding of these effectors to the enzyme. When these curves are transformed into straight lines by use of the modified Hill equation, the slopes (n) are greater than 1. Since n is an interaction coefficient (25, 26), values of n greater than 1 indicate the presence of at least two (possibly identical) interacting binding sites on the enzyme for these effectors. The similarity of the pH dependence of histidine and thiazolealanine inhibition suggests that the two inhibitors

are acting at similar if not identical binding sites on the enzyme. Sigmoid curves for histidine inhibition have also been reported (36) for partially purified *E. coli* phosphoribosyltransferase.

Evidence for the existence of more than one type of binding site comes from two quarters. The feedback-resistant mutant, *hisG1109 hisIF135*, requires a higher concentration of histidine to protect it during the heat step than does the wild type enzyme. This suggests that histidine is still capable of binding to the feedback-resistant enzyme, thereby stabilizing it to thermal inactivation. Second, Martin (3) has shown that ^{3}H-L-histidine binds equally well to histidine-sensitive and to histidine-insensitive enzyme. It cannot be assumed that the histidine binding site associated with stabilization or binding in these studies is necessarily required for feedback inhibition, although such an explanation is attractive.

These observations, together with the results of the Hill plots, can be explained by postulating that histidine inhibition depends upon the binding of histidine to at least two interacting binding sites. Thus, whereas binding of histidine is necessary for feedback inhibition, it is not sufficient unless binding to at least a second site occurs, and there is some interaction between the two sites.

The feedback-resistant enzyme was purified in order to find any difference in the response of this enzyme to histidine. The purified wild type and feedback-resistant enzymes were found to have similar sedimentation coefficients, subunit molecular weights, and tryptic peptide maps. The two enzymes, therefore, are quite similar in their physical and chemical properties. Since the feedback-resistant mutation lies in the operator-proximal third of the *G* gene (37), it is likely that the mutation is a missense, rather than a nonsense or frameshift, mutation. Mutations of the latter two types would produce a truncated protein (13, 37, 39). Therefore, the two enzymes probably differ only by the substitution of a single amino acid.

The most striking divergence between the wild type and feedback-resistant enzymes was in the response of their individual difference spectra to L-histidine. The difference spectrum of the wild type enzyme was still increasing in absorbance at 280 mμ 170 min following the addition of histidine. In contrast, no change in the difference spectrum of the feedback-resistant enzyme occurred upon the addition of histidine. The fact that the difference spectrum of the feedback-resistant enzyme remained constant makes it unlikely that there is a trivial explanation for the change in the difference spectrum of the wild type enzyme. These findings are consistent with the occurrence of a conformational change in the enzyme when histidine interacts with the intact feedback inhibition sites possibly followed by precipitation of the wild type enzyme. Presumably in the feedback-resistant enzyme, these sites are altered or interaction with histidine does not produce the conformational change (or both). It has recently been shown that binding of histidine to the wild type phosphoribosyltransferase is accompanied by a conformational change in the enzyme in which 12 previously exposed tyrosyl residues become buried.[10]

Finally, on the basis of a molecular weight of 215,000 for the native enzyme, a subunit molecular weight of 35,000, and an amino acid analysis, Voll *et al.* (4) calculated that, if the phosphoribosyltransferase were composed of six identical subunits of 35,000 molecular weight, tryptic digests of the enzyme would give 39 fragments. These authors reported that a preliminary tryptic peptide map of the digested protein gave 34 to 42 tryptic fragments plus a small amount of core material. The tryptic peptide maps of the wild type phosphoribosyltransferase reported in this paper were reproducible and gave 34 to 36 ninhydrin-positive spots plus a small amount of core material. A subunit molecular weight of approximately 34,000 was obtained in SDS polyacrylamide gel electrophoresis. These results in conjunction with the earlier observations of Voll *et al.* (4) indicate that the wild type and feedback-resistant phosphoribosyltransferases are composed of six similar subunits.

[10] Drs. F. Blasi, S. M. Aloj, and R. F. Goldberger, personal communication.

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Purification and Properties of the Wild Type and a Feedback-resistant Phosphoribosyladenosine Triphosphate: PYROPHOSPHATE PHOSPHORIBOSYLTRANSFERASE, THE FIRST ENZYME OF HISTIDINE BIOSYNTHESIS IN SALMONELLA TYPHIMURIUM

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