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 nm upon the addition of L-histidine, whereas that of the feedback-resistant enzyme remained constant following the addition of L-histidine.

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) in 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 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 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 concentrations stated are based on the results of an orotidine 5'-phosphate pyrophosphorylase assay for PP-ribose-P done by the manufacturer. 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hisT1504 inoculum after 16 hours of growth was 900 g (wet weight) of cells. The fermenter yield of cells from a l-liter excess histidine (12). The fermenter yield of cells from a l-liter

These conditions result in up to a 25-fold derepression of the his-zenzymes (7). The

of the histidine operon, are constitutive for the histidine biosyn-

30.fold derepression of the phosphoribosyltransferase in constitu-

ative 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

the wild type enzyme.3 The feedback-resistant phosphoribosyl-

transferase was isolated from strain hisGIl hisIF (5).

Bacterial Strains

The wild type phosphoribosyltransferase was isolated from strain hisT1504 hisC146, 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.4 The feedback-resistant phosphoribosyltrans-

ferase was isolated from strain hisG7109 hisIF135 (5). Growth of Bacterial Strains

Cells were cultured in a 300-liter fermenter at 37° and har-

vested 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 so-

dium citrate 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, hisG7109 hisIF135, was grown in the fermenter on Medium E (11) containing 0.5% glucose, 0.06 mm histidinol, 0.0075 mm r-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 con-

version of the product of the forward reaction, PR-ATP, to 

BBM III (1). BBM III has an extinction coefficient of 8.0 x

10^6 M^-1 cm^-1 at 290 mp (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 Tri-

HCl buffer (pH 8.5), 3 μmoles of MgCl2, 45 μmoles of KCl, 1.5 μmoles of ATP, 0.15 μmole of PR-ribose-P, 5 μl of an extract of strain hisG70 that had been passed through a Sephadex G-50 column (19), and enzyme in a final volume of 0.3 ml. To initiate the reaction, PR-ribose-P was added and the initial change in absorbance at 290 μm 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 μm of 0.10 per 5 min. Based upon the extinction coeffi-

cient of BBM III (12), 1 unit of activity corresponds to the forma-

tion of 0.75 n mole 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 μm. Pyrophosphatase Assay—The assay measures the formation of PR-ATP at 290 μm 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 PR-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 μmoles 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 μm of PR-ATP. Neither ATP nor PR-ATP absorbs appreciably at 290 μm (1). The reaction mixture contained 10 μmoles of Tri-HCl buffer (pH 8.5), 0.3 μmole of tetrasodium pyrophosphate, 6.8 μmoles of PR-ATP, 0.5 μmole of MgCl2, 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 μm 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 μmoles 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, Vinuela, and Maizel (15).
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 Voil, Appella, and Martin (4) at pH 8.6 and containing 0.01 M β-mercaptoethanol. The temperature was 25°C.

Enzymatic Synthesis of PR-ATP and PR-AMP

PR-ATP and PR-AMP were generated in a cell-free extract of mutant hisl504 hisWAF1232 (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 A280/A299 ratio of 5.32 at pH 8.5, the PR-AMP had an A560/A299 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

Lyophilized 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°C for 5 hours. Following lyophilization of the trypsin 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 Rynear (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). The nitrogen determinations were generously performed by Dr. W. C. A1ford 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 diithiothreitol. 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°C 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 Blender 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 x 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°C was attained in 5 min by immersion of the flask, with vigorous agitation, into a water bath at 70°C. The temperature of the contents of the flask was maintained at 61°C by agitation in a 61°C water bath for 8 min. Rapid cooling to 5°C was achieved by immersion of the flask in an ice-salt bath. A supernatant fraction was obtained after centrifugation at 27,000 x 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 x 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 x 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 x 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 standard. 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 x 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
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. The concentrated enzyme solution was then made 3 mM in NaCl 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 6800 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 an extensive dialysis against distilled water. The nitrogen content and the percentage weight nitrogen in the enzyme (18%) were 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 specific activity 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 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 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 showed one major band and one minor band (see below).

**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.

**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 hisP128 (5), in which the feedback inhibition site of the enzyme is insensitive to histidine.

A specific activity of 6600 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 an 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.

<table>
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<th>Procedure</th>
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* Determined by the method of Lowry et al. (20).
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 nm per 5 min. The magnesium to ATP ratio was constant at 2:1. Symbols: •, 1.48 mM ATP; △, 2.99 mM ATP; □, 0.97 mM ATP. Inset, secondary plot of the ordinate intercepts against \(1/([\text{fixed substrate, ATP}]\) 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 nm per 5 min. The magnesium to ATP ratio was constant at 2:1. Symbols: •, 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 \(1/([\text{fixed substrate, PP-ribose-P}]\) of 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 nm per 5 min. A, reciprocal velocity against reciprocal PP-ribose-P (PRPP) concentration at various L-histidine concentrations. Symbols: •, no L-histidine; △, 3.3 × 10^{-4} M L-histidine; ■, 6.7 × 10^{-4} M L-histidine. B, reciprocal velocity against reciprocal ATP concentration. Symbols: •, no L-histidine; △, 3.3 × 10^{-4} M L-histidine; ■, 6.7 × 10^{-4} 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 \(5.6 \times 10^{-4}\) M (Fig. 2). The apparent \(K_m\) for ATP (with the standard PP-ribose-P concentration) is \(4.3 \times 10^{-4}\) 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 \(1/([\text{fixed substrate}]\) were drawn (Figs. 2 and 3, insets). The concentration-independent Michaelis constant for ATP is \(4.2 \times 10^{-4}\) M (Fig. 2, inset), while the concentration-independent Michaelis constant for PP-ribose-P is \(1.6 \times 10^{-4}\) 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 \(5.1 \times 10^{-4}\) M (Fig. 4A) and that for ATP (standard PP-ribose-P concentration) was \(4.8 \times 10^{-4}\) M (Fig. 4B).

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 concentration at various L-histidine concentrations. Symbols: •, no L-histidine; △, 3.3 × 10^{-4} M L-histidine; ■, 6.7 × 10^{-4} M L-histidine. PRPP, PP-ribose-P.
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.

<table>
<thead>
<tr>
<th>Reaction mixture</th>
<th>Enzyme activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete</td>
<td>324 units/ml</td>
</tr>
<tr>
<td>Minus phosphoribosyltransferase</td>
<td>0</td>
</tr>
<tr>
<td>Minus PR-ATP</td>
<td>0</td>
</tr>
<tr>
<td>Minus PPi</td>
<td>0</td>
</tr>
<tr>
<td>Minus Mg++</td>
<td>0</td>
</tr>
<tr>
<td>Minus PR-ATP, plus PR AMP</td>
<td>0</td>
</tr>
<tr>
<td>Plus L-histidine</td>
<td>137 units/ml</td>
</tr>
</tbody>
</table>

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-AMP and PPi 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 x 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 x 10^-4 M. In another preparation, 8 x 10^-3 M L-histidine inhibited activity by 50%. Previously reported preparations of the purified enzyme were inhibited 50% by 6 x 10^-3 M and 9 x 10^-3 M L-histidine (4).

When the PP-ribose-P concentration was varied (Fig. 4A), L-histidine was an uncompetitive inhibitor altering both Vmax.
Inhibition by L-histidine and thiazolealanine inhibition on pH. The pH of the assay mixture was measured at the end of each assay.

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

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 percent inhibition occurred at $4 \times 10^{-4} M$ 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 $\alpha$-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 nm of the wild type enzyme upon the addition of L-histidine (Fig. 11). The absorbance at 280
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 Ti 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 (30) 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, hisG1100 hisF135, 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 (9) has shown that '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 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 mU 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 30,000, 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 ninyhdrin-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.

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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

Harvey J. Whitfield, Jr.


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