Purification and Composition Studies of Phosphoribosyl-adenosine Triphosphate: Pyrophosphate Phosphoribosyltransferase, the First Enzyme of Histidine Biosynthesis

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SUMMARY

Highly purified preparations of N-1-(5'-phosphoribosyl)adenosine triphosphate: pyrophosphate phosphoribosyltransferase from Salmonella typhimurium have been obtained. The phosphoribosyltransferase is the first enzyme unique to histidine biosynthesis and is sensitive to feedback inhibition by histidine. The procedure used for purification preserved the histidine sensitivity of the enzyme. A molecular weight of 215,000 for native enzyme was obtained. In high concentrations of guanidine.HCl, the enzyme dissociated into several, perhaps identical, subunits. An amino acid analysis of one preparation is presented. Methionine has been detected as an NH₂-terminal amino acid in the enzyme.

N-1-(5'-Phosphoribosyl)adenosine triphosphate: pyrophosphate phosphoribosyltransferase, formerly called phosphoribosyl-ATP pyrophosphorylase, catalyzes the first step of the pathway for histidine biosynthesis in Salmonella typhimurium (1).

ATP + 5-phosphoribosyl 1-pyrophosphate → N-1-(5'-phosphoribosyl)-ATP + pyrophosphate

The product of the reaction, PR-ATP, is converted by a series of nine enzymatic steps to histidine.

The genes which specify the enzymes of histidine biosynthesis in Salmonella constitute a single operon which is regulated by the amount of internal histidyl-transfer ribonucleic acid in the cell (see review by Ames et al. (2)). The phosphoribosyltransferase is of particular interest because it is the object of end product inhibition by histidine (1, 3) and because it is coded for by the gene immediately adjacent to the operator region of the operon.

The phosphoribosyltransferase was partially purified by Martin (3). Highly purified preparations retaining full sensitivity to histidine inhibition have now been obtained. The molecular weight and subunit structure of the purified enzyme were investigated. Earlier estimates (3, 4) of a molecular weight of about 200,000 for the enzyme have been confirmed, and evidence is presented that the enzyme is composed of subunits.

EXPERIMENTAL PROCEDURE

Chemicals—Sephadex G-50, G-150, and G-200 were purchased from Pharmacia; DEAE-cellulose was obtained from Schleicher and Schuell. Urea (Mallinckrodt Chemical Company) was recrystallized from 95% ethanol. Guanidine-HCl (Aldrich) was recrystallized from ethanol. 1-Fluoro-2,4-dinitrobenzene-¹⁴C (uniformly labeled, 4.37 μCi per μmole) and tritiated 1-fluoro-2,4-dinitrobenzene (3, 5, 6, 8H, 112 μCi per μmole) were obtained from Nuclear-Chicago. Dinitrophenyl-amino acid derivatives and ammonium sulfate (special enzyme grade) were obtained from Mann.

Bacterial Strains—The phosphoribosyltransferase was isolated from extracts of the histidine-requiring mutants, his El1 and his El2 of Salmonella typhimurium, strain LT-2. His El1 is a point mutant in the "E" gene. His El2 is a deletion mutant missing all but the three genes at the operator end of the operon. Both mutants are deficient in the enzyme, PR-ATP pyrophosphohydrolase, which is specified by the "E" gene, and which converts PR-ATP to the next intermediate, PR-AMP (5).

The mutants were grown in a 300-liter fermentor as described by Margolies and Goldberger (6), under conditions which result in up to a 25-fold derepression of the histidine biosynthetic enzymes (7). His El1 was grown in the minimal medium of Vogel and Bonner (8) containing 0.05 mM histidinol, 0.0075 mM histidine, 0.4 mM adenine, and 0.5% glucose as the carbon source. His El2 was grown in the same medium minus adenine and with 0.15% glucose as the carbon source.

Enzyme Assay—The phosphoribosyltransferase activity was assayed by the method of Ames, Martin, and Garry (1) which is based on the high extinction coefficient of PR-AMP at 290 μm. The reaction mixture contained 30 μmoles of Tris-HCl buffer, pH 8.5, 3 μmoles of MgCl₂, 45 μmoles of KCl, 0.1 μl of yeast inorganic pyrophosphatase (crystallized twice, Worthington), 1.5 μmoles of ATP, 0.15 μmole of PP-ribose-P, and enzyme in a final volume of 0.30 ml. PP-ribose-P was added to start the reaction and the initial change in absorbance at 290 μm at 27 +
1° was followed on a Beckman spectrophotometer equipped with a Gilford multiple sample absorbance recorder. When enzyme was omitted from the reaction mixture, no appreciable change in absorbance at 280 m\textmu\. occurred.

A unit of activity is defined as a change in absorbance of 0.10/5 min which corresponds to the transformation of 0.00167 \mu\textnumero of substrate per min and is equivalent to 10.4 units as defined by Martin (3).

The reaction mixture used differs from that previously described (1, 3) by the inclusion of KCl. KCl was added to the reaction mixture since it was found to stimulate phosphoribosyltransferase activity in crude extracts. At a concentration of 0.13 m, KCl increased the reaction rate by about 40\%. A number of other salts, e.g. ammonium sulfate, ammonium chloride, and sodium sulfate, showed the same effect at the same concentration. Sodium chloride gave less stimulation of activity. Increasing the concentration of KCl in the reaction mixture to 0.20 m did not result in any further stimulation of activity.

Polyacrylamide Disc Gel Electrophoresis—Electrophoresis of the enzyme was done in polyacrylamide gels (pH 9.5) essentially as described by Davis (9), except that 50\% urea (w/v) was added to both upper and lower gels to give a final concentration of 6.0 m.

Ultracentrifugation—Sedimentation velocity and equilibrium studies were done in a Beckman model E analytical centrifuge at 20°. Equilibrium studies were performed by the meniscus depletion method of Yphantis (10) with a column height of 3 mm.

Amino Acid Analysis—Enzyme was dialyzed against 1 m sodium bicarbonate, lyophilized to dryness, and taken up in constant boiling HCl. Samples were dehydrated by freezing and thawing under vacuum, sealed under vacuum, and hydrolyzed for varying lengths of time at 105°. After hydrolysis, the samples were dried over NaOH in an evacuated desiccator. Amino acid analyses were carried out with a Beckman amino acid analyzer, model SP 144.

Performic acid oxidation of the enzyme was performed at 0° according to the method of Schram, Moore, and Bigwood (11). The oxidized protein was recovered by freeze drying and then hydrolyzed for 24 hours as described above.

Nitrogen Determination—Enzyme was assayed for nitrogen content by the micro-Kjeldahl technique. Analyses were carried out by the Analytical Chemistry Division of the National Institutes of Health by the method of Ma and Zuazaga (12).

NH\textsubscript{2}-terminal Amino Acid Determination—A modification of the Sanger method (13) which employed radioactive fluorodinitrobenzene was used to detect NH\textsubscript{2}-terminal amino acids in purified enzyme preparations. Before reacting with fluorodinitrobenzene, enzyme preparations were dialyzed against 1.0 m sodium bicarbonate for 24 hours at 4°. The reaction mixture contained, in a final volume of 0.20 ml, approximately 1 \mu\textnumeros of enzyme, approximately 200 \mu\textnumero of \textsuperscript{14}C-FDNB (0.05 m of an alcoholic solution), and 20 \mu\textnumero of sodium bicarbonate. In some cases urea was added to the reaction mixture at a concentration of 5.0 m. The dinitrophenylation reaction was allowed to proceed for 3\1/2 hours in the dark. Then 1.0 ml of 1 N HCl was added, and untreated reagent was extracted with ether saturated with 1 N HCl. When urea was not present in the reaction mixture, the samples were lyophilized to dryness preparatory to hydrolysis. When urea was present, it was removed before hydrolysis by precipitating the dinitrophenylated protein with 5\% trichloroacetic acid with insulin as a carrier protein. The precipitate was washed twice with trichloroacetic acid and twice with ether and dried in air. The dry samples were taken up in 0.5 ml of constant boiling HCl and deaerated by freezing and thawing under vacuum. Hydrolysis in evacuated sealed tubes was carried out at 105°. After hydrolysis the samples were dessicated to dryness, taken up in 1.0 ml of 1 N HCl, and extracted with ether. Chromatography was performed on the ether phase along with known DNP-amino acid derivatives on Whatman No. 4 paper. Chromatography was carried out in one dimension with the \textit{t}-amyl alcohol-phthalate system of Blackburn and Lowther (14). The sample strip was cut from the chromatogram and passed through a Vanguard automatic chromatogram scanner to detect regions of radioactivity. Radioactive peaks corresponding to dinitrophenol and dinitroaniline (known degradation products produced by hydrolysis) and to N-DNP-methionine were found. The portion of the sample strip corresponding to N-DNP-methionine was sectioned and the sections counted for radioactivity in a Nuclear-Chicago gas flow counter along with a section of Whatman No. 4 paper of similar size and saturated with a known amount of the \textsuperscript{14}C-FDNB reagent. By comparing the counts per min given by the chromatogram sections corresponding to DNP-methionine with the counts per min given by the FDNB standard, an estimate of the amount of DNP-derivative per mole of enzyme treated was obtained.

In one experiment, methionine controls were run. A known amount of methionine, with and without added protein (see “Results”), was reacted with radioactive FDNB in the absence of urea as described above. At the end of the reaction the excess FDNB was converted to dinitroaniline by adding a 5-fold molar excess of ammonium bicarbonate. Then 1.0 ml of 1 N HCl was added, and the samples were lyophilized to dryness, hydrolyzed, and further treated as described above. In this experiment, tritiated FDNB was used as the reagent, and chromatogram sections were counted in toluene phosphor solution in a Packard Tri-Carb scintillation counter.

Protein Determination—Protein concentration was determined by the method of Lowry et al. (15), with insulin as a standard. For the determination of the protein concentration of purified enzyme preparations see “Results.”

RESULTS

Enzyme Purification

Standard Buffer—The phosphoribosyltransferase is a very labile enzyme. \textit{\textbeta}-Mercaptoethanol and NaCl help to stabilize enzymatic activity (3). Histidine at an intermediate concentration protects the enzyme from heat inactivation (3). Therefore, a buffer containing \textit{\textbeta}- mercaptoethanol, NaCl, and histidine of the following composition was employed: 0.01 m Tris, 0.10 m NaCl, 0.4 m histidine, 2.8 m \textit{\textbeta}-mercaptoethanol, and 0.5 m EDTA. The pH was adjusted to 7.5 with HCl. \textit{\textbeta}-Mercaptoethanol was added to the buffer immediately before use. Extracts prepared in this buffer and stored at 4° remained fully active for at least 3 days. This buffer was used throughout the purification procedure with generally no significant losses of enzyme activity.

Preparation of Extracts—Two methods were used in preparing extracts. For less than 200 g of cells, wet weight, the cells were resuspended in 2.5 to 3.0 volumes of buffer and subjected to sonic disruption in 40-ml aliquots in a Branson S-75 sonifier for
soft pellet material was recovered and centrifuged at 31,000 × g. Generally, the extracts were not fully clarified in this step. The homogenate was centrifuged at 10,000 × g for 60 min. Gen-

40 see at setting 6. The extracts were centrifuged in a Servall RC-2 centrifuge at 31,000 × g for 20 min and the supernatant solution was recovered.

For more than 200 g of cells, wet weight, the cells were re-
suspended in 1.5 to 2.0 volumes of buffer and passed once through a Gaulin Laboratory homogenizer (Monton-Gaulin Manufacturing Corporation, Everett, Massachusetts) at 8,000 p.s.i. The homogenate was centrifuged at 10,000 × g for 60 min. Generally, the extracts were not fully clarified in this step. The upper clear supernatant fluid was decanted, and the remaining soft pellet material was recovered and centrifuged at 31,000 × g for 20 min. The clear supernatant fluid was decanted and combined with the supernatant from the first centrifugation to form the extract.

Sonic disruption was carried out with the container of bacterial suspension immersed in an ice bath. Homogenization was done at room temperature, and the homogenate was collected into an iced container. Other operations and subsequent procedures were carried out at 0–4° unless noted.

The extracts contained about 10 mg of protein per ml. Protein (and enzyme activity) was determined on an aliquot of the extract which had been passed through a Sephadex G-50 column equili-

brated with the standard buffer mixture at pH 8.0. The total volume of solution of the enzymatic activity.

In the purification presented in Table I, the supernatant solution obtained after Mn++ treatment had considerable activity which was recovered in the further precipitate which formed after standing at 4° overnight. This led to a smaller increase in specific activity than was usually obtained because of a greater recovery of protein in the precipitate.

In some cases, the supernatant solution still contained a considerable portion (20% or more) of enzymatic activity after this amount of time. Activity remaining in the supernatant solution could be recovered upon the addition of 0.005 volume more of 1 M MnCl₂ and further standing.

The precipitate was suspended in one-half the original volume of buffer plus 0.015 volume of 1.0 M Tris base and worked into solution. Material which failed to go into solution was devoid of enzyme activity and was removed by centrifugation at 10,000 × g for 20 min. The Tris base added to the buffer raised the pH of the suspension to about 8.0 and was necessary to effect solution of the enzymatic activity.

Generally 80% or more of the input activity was recovered in the Mn++ step with only about 20% of the input protein being precipitated.

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**Table I**

<table>
<thead>
<tr>
<th>Step</th>
<th>Volume</th>
<th>Activity</th>
<th>Protein*</th>
<th>Specific activity</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extract</td>
<td>645</td>
<td>2.52 × 10⁶</td>
<td>3220</td>
<td>48</td>
<td></td>
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<tr>
<td>Mn++ precipitation</td>
<td>335</td>
<td>3.02 × 10⁶</td>
<td>2460</td>
<td>122</td>
<td>120</td>
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<tr>
<td>DEAE-cellulose</td>
<td>227</td>
<td>2.45 × 10⁶</td>
<td>613</td>
<td>400</td>
<td>97</td>
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<tr>
<td>Second DEAE-cellulose</td>
<td>145</td>
<td>1.59 × 10⁶</td>
<td>324</td>
<td>599</td>
<td>63</td>
</tr>
<tr>
<td>Ammonium sulfate</td>
<td>9.5</td>
<td>1.90 × 10⁶</td>
<td>211</td>
<td>902</td>
<td>75</td>
</tr>
<tr>
<td>Sephadex G-150</td>
<td>15</td>
<td>3.5 × 10⁶</td>
<td>14.5</td>
<td>2400</td>
<td>14</td>
</tr>
</tbody>
</table>

* Determined by the method of Lowry et al. (15).

**Ammonium Sulfate Fractionation**—Ammonium sulfate, 22 g/100 ml of the combined DEAE-cellulose fractions, was added. The ammonium sulfate was added slowly with stirring. At intervals, sufficient 1.0 M Tris base was added to keep the pH neutral to slightly alkaline. The solution was kept at 4°C for 45 min and centrifuged at 10,000 × g for 10 min. The precipitate was discarded and 12.5 g of ammonium sulfate per 100 ml of original volume were added to the supernatant solution as described above. The resulting precipitate which contained most of the input activity was resuspended in 0.05 to 0.10 of the original volume of buffer. Generally a 2-fold purification was achieved.

Under favorable conditions the first three steps brought the specific activity to about 1000 units per mg of protein with a 60% or greater recovery of the activity in the extract.

**Sephadex Chromatography**—The ammonium sulfate fraction was applied to a molecular sieve column. Either Sephadex G-200 or G-150 was used. The dry gel was swollen in 0.10 M NaCl, poured, and washed with the standard buffer. In some cases, the gel was poured in an excess of buffer, and washing was omitted. Since the ammonium sulfate fraction was of higher density than the buffer, either the NaCl concentration of the column and eluent buffer was raised to 0.20 M or a layer of Sephadex G 25 (coarse) was added to the top of the column.
large purification. The elution profile of active protein
fractionation and the resulting slurry was dialyzed for 84 hours in 2
amount of buffer was added to the pellet from the second precipi-
tationated with ammonium sulfate as described above. A small
activity were combined and had a 4.5-fold increase in specific
activity in crude extracts. In the present study, KCl was
inactivated at the higher protein concentrations. The negative of the
elution profile with ammonium sulfate, a second passage of the
combined fractions through Sephadex G-200 or G-150 generally
yielded fractions containing highly purified enzyme with specific
activities of 200 to 2400 units per mg of protein. The presence
of proteins with mobilities on Sephadex similar to that of the
phosphoribosyltransferase led to a rather low recovery of the
enzyme in this step (Table I).

Large Scale Purification—To obtain a greater yield of protein
the following procedure was used. In 2 liters of buffer, 1 kg of
cells, wet weight, was resuspended, homogenized, and centrifuged.
The supernatant solution was diluted 4-fold with distilled water.
The pH was adjusted to 7.5 with Tris, and the solution was
applied directly to a 2-liter DEAE-cellulose column. The col-
umn was washed with 4 bed volumes of buffer containing 0.05 M
NaCl and then eluted with 8 liters of a NaCl gradient (0.05 M
to 0.50 M). Fractions containing the major portion of eluted
activity were combined and had a 4.5-fold increase in specific
activity over the extract. The combined fractions were frac-
tionated with ammonium sulfate as described above. A small
amount of buffer was added to the pellet from the second precipi-
tation and the resulting slurry was dialyzed for 84 hours in 2
liters of buffer. The dialyzed material was centrifuged to remove
the slight precipitate which formed on dialysis and was then
applied in a final volume of 40 ml to a 4.3-liter Sephadex G-150
column. Fractions containing 80% of the input activity were
combined and represented a 4-fold purification over the DEAE-
cellulose step. A second DEAE-cellulose chromatography,
followed by two passages through Sephadex G-200, yielded a
highly purified preparation. The elution profile of active protein
from the second Sephadex G-200 column is shown in Fig. 1.
Fractions 56 through 65, having approximately constant specific
activity, were combined to form the final preparation (Prepara-
tion of February 1966). The yield was 52 mg of protein.

The activity in this preparation was more unstable than usual.
It was sensitive to dilution. When assayed undiluted in the
presence of p-mercaptoethanol (see "Stability" under "Prop-
erties of Purified Enzyme") the highest specific activity obtained
was 1400 units per mg of protein.

Properties of Purified Enzyme Preparations

Stability—Purified enzyme preparations generally began to
lose activity soon after isolation but retained 50% or more of
their activity for several days in standard buffer at 4°C. Addition
of 14.3 μmoles of β-mercaptoethanol to the assay mixture gen-
erally led to a significant increase in activity of partially inactive
preparations but had no effect on fully active preparations.
Under the conditions of the enzyme assay, this amount of β-
mercaptoethanol gives a small but significant increase in absorption
at 290 nm with time in the absence of enzyme and substrates.
Therefore, in determining rates in the presence of β-mercap-
toethanol, PP-ribose-P was not added until the rate due to β-mer-
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Histidine Inhibition—Martin (3) reported that 6 ± 2 × 10^{-5}
m histidine gives a 50% inhibition of phosphoribosyltransferase
activity in crude extracts. In the present study, KCl was
included in the reaction mixture. The presence of KCl de-
creased somewhat the histidine sensitivity of the phosphoribosyl-
transferase, 8.0 ± 0.1 × 10^{-5} M histidine being required to give a
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6.0 × 10^{-5} M for the Preparation of November 1965 and 9.0 × 10^{-5}
M for the Preparation of February 1966 (assayed in the presence
of β-mercaptoethanol).

Sedimentation Velocity The sedimentation pattern of the
Preparation of February 1966 is shown in Fig. 2. Protein
sedimented as a single symmetrical peak with an s20, w of 8.83 S.
Another purified preparation (Preparation of June 1965) at a
concentration of 6.0 mg of protein per ml gave an s20, w of 8.42 S.
These values are somewhat lower than those (s20, w = 9.5 S) pre-
viously obtained with less pure preparations (3, 4).

Disc Gel Electrophoresis—When enzyme preparations, judged
to be highly purified by other criteria (elution from Sephadex and
ultracentrifugation analysis) were subjected to electrophoresis
on polyacrylamide gels, multiple band patterns were obtained.
It was thought that these results could have been caused by
aggregation of enzyme protein in the gel. Since such aggregation
might be prevented in high concentrations of urea, enzyme
samples and gels were made to 6.0 M in urea. Under these
conditions only one darkly staining band was observed (Fig. 3).
Small amounts of what may be contaminant bands were observed
at the higher protein concentrations. The negative of the

![Fig. 1. Elution profile of the Preparation of February 1966 on the second Sephadex G-200 column. Enzyme in a volume of 1.8 ml was applied to a 300-ml Sephadex G-200 column. The column was eluted with the standard buffer, and fractions of 2.5 ml in volume were collected and assayed for phosphoribosyltransferase activity and absorption at 280 nm until no more enzymatic activity eluted.](http://www.jbc.org/content/1763/f1)

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Fraction Number
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FIG. 2. Sedimentation velocity pattern of purified enzyme. The Preparation of February 1966, at a concentration of 2.8 mg of protein per ml in the standard buffer, was sedimented at 20° and 59,780 rpm. Pictures were taken at 8-min intervals. A sedimentation constant, $s_{20,w}$, of 8.83 S was calculated from the data. The above picture was taken approximately 1 hour after reaching full speed.

The percentage weight of nitrogen of the enzyme, as determined from the amino acid composition, was 18%.

An extinction coefficient for the enzyme was determined. The nitrogen content of purified enzyme was determined on two separate preparations. Samples of the Preparations of November 1965 and February 1966 were dialyzed against 10 or 1 mM sodium bicarbonate, and measured aliquots were assayed for nitrogen by the micro-Kjeldahl technique. The nitrogen content of the samples was equated with the percentage weight of nitrogen in the enzyme (18%) to obtain the protein concentration. The same samples were measured for absorbance at 280 μM (1-cm path length) and for protein by the method of Lowry et al. (15). The protein concentration of both preparations as determined by nitrogen analysis was 1.21 times greater than that determined by the method of Lowry et al. with insulin as a standard. An absorbance of 1.0 at 280 μM corresponded to 1.34 mg of protein per ml for both samples as determined by nitrogen analysis.

Molecular Weight of Native Enzyme—In previous studies, with impure preparations, a molecular weight for the enzyme of roughly 200,000 was obtained by sedimentation studies in sucrose density gradients (3, 4). In this study, molecular weight was determined on purified enzyme by equilibrium centrifugation with the meniscus depletion method of Yphantis (10). Enzyme

photograph shown in Fig. 3 was traced with a Joyce-Loebl microdensitometer, and the areas under the peaks were calculated for the three highest protein concentrations. The major band represented 75 to 80% of the total area, the remaining area corresponding to five bands of roughly equal intensity. Since the minor components may well represent aggregate forms of the enzyme not disassociated by the urea treatment, this estimate of purity is a minimum figure.

Composition Studies—Purified enzyme (Preparation of February 1966) was analyzed for amino acid composition after 48, 72, and 96 hours of acid hydrolysis. Cysteine was determined separately by amino acid analysis of performic acid-oxidized enzyme. The tryptophan content was not determined. The results are presented in Table II. The average number of micromoles of amino acid per 100 mg of protein (last column, Table II) was calculated from the values obtained at the three different times of hydrolysis, except for threonine and serine. The values for threonine and serine were obtained by extrapolation to zero time of hydrolysis. From the average amino acid content, a partial specific volume for the enzyme of 0.747 cm³ per g was calculated as described by Cohn and Edsall (16).

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FIG. 3. Disc gel electrophoresis of purified enzyme. Samples (0.10 ml) of the Preparation of October 1965 in standard buffer plus 50%, w/v, urea were applied to polyacrylamide gels made up to 6.0 M in urea and electrophoresis was performed at 4°. Approximately 27, 43, 8, and 3 μg of protein (left to right) were applied. Gels were stained for protein with a 0.2% solution of Amido Schwarz (Allied Chemical) in 7% acetic acid.
in the standard buffer mixture was centrifuged to equilibrium at 12,590 rpm and 20° for 72 hours. Two separate enzyme preparations were sedimented, and molecular weights were calculated with a \( \varepsilon \) of 0.747 cm\(^3\) per g determined from the amino acid analysis.

One preparation (Preparation of October 1965) analyzed at an initial concentration of 0.95 mg of protein per ml yielded a weight average molecular weight of 217,000. Another preparation (Preparation of February 1966) was analyzed at initial concentrations of 0.71 and 1.42 mg of protein per ml. Weight average molecular weights of 221,000 and 210,000, respectively, were obtained. A plot of ln \( C \) against \( x^2 \) for the Preparation of February 1966 at an initial concentration of 0.71 mg of protein per ml is shown in Fig. 4. The linearity of the curve suggests a high degree of homogeneity.

**Molecular Weight of Enzyme in Guanidine·HCl—**Molecular weight studies of the phosphoribosyltransferase in high concentrations of guanidine·HCl indicate that the enzyme is composed of several subunits. Enzyme preparations were dialyzed against a 250-fold excess of 5.0 M guanidine·HCl and 0.143 M \( \beta \)-mercaptoethanol for 7 days. Sedimentation velocity and sedimentation equilibrium studies were then performed.

Enzyme (Preparation of June 1965) at a concentration of 0.9 mg of protein per ml was sedimented for 2 hours at 50,740 rpm and 20°. Pictures were taken at 8-min intervals. An \( s_{20,w} \) of 0.94 S was calculated. This value was corrected to a solvent with a density of H\(_2\)O at 20° with the following data: a density of 1.1265 g per ml for the guanidine·HCl solution determined from the index of refraction and the data of Kielley and Harrington (17); a viscosity of 1.16 centipoise determined by viscometry; and a partial specific volume of 0.747 cm\(^3\) per g. An \( s_{20,w} \) of 1.75 S was obtained as compared with an \( s_{20,w} \) of about 8.6 S obtained for native enzyme.

The same preparation of denatured enzyme was centrifuged to equilibrium as described above. Centrifugation at 35,600 rpm and 20° was continued for 27 hours after reaching full speed. On the basis of a \( \varepsilon \) of 0.747 cm\(^3\) per g, a weight average molecular weight of 36,000 was calculated.

Another preparation (Preparation of October 1965) at an initial concentration of 1.5 mg of protein per ml was centrifuged at 35,600 rpm and 20° for 70 hours. The coordinates are as stated under Fig. 4.

![Fig. 4. Equilibrium centrifugation analysis of native enzyme.](http://www.jbc.org/)

**Table II**

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Hydrolysis for 48 hrs</th>
<th>Hydrolysis for 72 hrs</th>
<th>Hydrolysis for 96 hrs</th>
</tr>
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<tr>
<td></td>
<td>( \mu ) moles/100 ( \mu ) protein</td>
<td>( \mu ) moles/100 ( \mu ) protein</td>
<td>( \mu ) moles/100 ( \mu ) protein</td>
</tr>
<tr>
<td>Lysine</td>
<td>50.3</td>
<td>46.9</td>
<td>49.0</td>
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<td>Histidine</td>
<td>15.9</td>
<td>16.5</td>
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<td>Arginine</td>
<td>61.2</td>
<td>59.6</td>
<td>60.2</td>
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<td>Aspartic acid</td>
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<td>Threonine</td>
<td>39.5</td>
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<td>Serine</td>
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<td>Glutamic acid</td>
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<td>114.2</td>
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<td>Proline</td>
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<td>Glycine</td>
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<td>Valine</td>
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<td>Methionine</td>
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<td>Tyrosine</td>
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<td>19.1</td>
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<td>21.2</td>
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<tr>
<td>Half-cystine</td>
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<td>21.1</td>
<td>21.2</td>
</tr>
<tr>
<td>Ammonia</td>
<td>114.6</td>
<td>119.1(^a)</td>
<td>129.8</td>
</tr>
</tbody>
</table>

\(^a\) Result of one determination; the other values are the average of two determinations on the same hydrolysate.
try with the use of the method of Tanford, Kawahara, and Lapanje (18) which is based on the observation that proteins behave as random coils in 6 M guanidine - HCl. A sample of enzyme (Preparation of February 1966) was lyophilized to dryness, taken up in 6.0 M guanidine - HCl and 0.14 M β mercaptoethanol, and dialyzed overnight in a 10 - fold excess of the solvent. The outflow times of dilutions of the enzyme solution, ranging in concentration from 5.22 to 1.74 mg of protein per ml, were measured at 25° in an Ostwald viscometer with an outflow time of approximately 5 min for the solvent. From the data, an intrinsic viscosity of 31.9 ml per g was determined. From the empirical relationship [η] = 0.684 n^0.67 (18) a chain length, n, of 309 residues was calculated. The average molecular weight of amino acid residues in the phosphoribosyltransferase is approximately 109, giving a molecular weight of the enzyme in guanidine - HCl of 38,700.

The subunit molecular weight of the enzyme was also determined by utilizing the relationship between molecular weight, viscosity, and sedimentation coefficient expressed in the Scheraga and Mandelkern equation (19). By using the intrinsic viscosity and sedimentation coefficient obtained for the enzyme in guanidine - HCl and a ρ' of 2.5 × 10^2 (20), a molecular weight for denatured enzyme of 33,700 was obtained.

**NH₂-terminal Amino Acid Determination**—A micromethod for end group analysis, based on the method of Sanger and employing radioactive FDNB, was used to detect NH₂-terminal amino acids in the enzyme (see “Experimental Procedure”). Enzyme was reacted with ¹⁴C-FDNB, the dinitrophenylated product was hydrolyzed, and the ether-soluble phase of the hydrolysate was chromatographed along with reference DNP - amino acids. The resulting chromatogram is shown. (The direction of chromatography is from right to left.) Reference DNP - amino acids were located by their color. DNP - derivatives in the sample were located by scanning (solid line). The radioactive peak running behind DNP - methionine is dinitrophenol. Sections of the sample strip in the area of DNP - methionine were directly counted.

In the methionine control samples, DNP-methionine moved just ahead of dinitrophenol. In addition to N-DNP-methionine, the methionine control sample containing plasma serum albumin also showed a radioactive peak corresponding to N-DNP-aspartic acid. (Bovine albumin has one NH₂-terminal amino acid, aspartic acid (21, 22).) The recovery of methionine as N-DNP-methionine in the control samples was 16% in the absence of protein and 23% in its presence. In the experimental sample, 0.66 mole of N-DNP-amino acid derivative was found per mole of enzyme treated.

The quantitative values achieved in these experiments cannot be considered exact because of the small amounts of material used, because of the closeness of the N-DNP-methionine and dinitrophenol peaks in chromatography, and because of the difficulty, as discussed by Fraenkel-Conrat, Harris, and Levy (23), of designing a precise control to measure the expected loss of DNP-amino acids. However, from the observation that about 90% of free methionine is recovered as the dinitrophenylated derivative in the procedure as used, and that 1 or close to 1 mole of N-DNP-methionine is recovered per mole of enzyme treated, it seems reasonable to conclude that there are several (perhaps as many as 6) NH₂-terminal methionine residues per enzyme molecule weighing 215,000.

**DISCUSSION**

Purification of the enzyme, N-1-(5′-phosphoribosyl)-ATP: pyrophosphate phosphoribosyltransferase, has been hampered by the marked instability of the enzyme. Partial stabilization of the enzymatic activity was achieved by storage of enzyme preparations in Tris buffer containing NaCl, β mercaptoethanol, histidine, and EDTA.

Highly purified preparations of the enzyme have now been obtained. These preparations consist of fractions exhibiting constant specific activity in the final gel filtration step. They give a single peak in sedimentation velocity analysis and appear runs at the front and is not shown in Fig. 6.) The 8-hour hydrolysis sample showed the same distribution of radioactivity upon chromatography. To estimate the actual amount of N-DNP-methionine recovered, sections of the chromatograms corresponding to N-DNP-methionine were directly counted along with a known amount of the FDNB reagent as described under “Experimental Procedure.” There is very good agreement between the peak obtained by direct counting and the position of the N-DNP-met-methionine standards (Fig. 6).

Based on the specific activity of the FDNB reagent but ignoring any losses encountered in hydrolysis, extraction, etc. (hence giving a minimum value) a value of 1 mole of N-DNP derivative per mole (215,000 mol wt) of enzyme treated was calculated for the 8- and 16-hour hydrolysis samples.
homogeneous in sedimentation equilibrium studies. The finding of a single NH₂-terminal amino acid also suggests the preparations are homogeneous. When the enzyme was analyzed by disc gel electrophoresis in 6.0 M urea, one major band representing 75 to 80% of the protein and five minor bands of roughly equal staining intensity were observed. Since electrophoretically different forms of the enzyme appeared on disc gel electrophoresis in the absence of urea (possibly isozymes or aggregates), it is suspected that some of the minor components observed in the presence of urea may also be isozymic or aggregated forms of the enzyme.

The subunit structure of the phosphoribosyltransferase is of special interest since this enzyme exhibits end product inhibition and is coded for by the first gene of the histidine operon. Analysis of the native, catalytically active enzyme by sedimentation equilibrium centrifugation yielded a molecular weight of 215,000. In guanidine·HCl, the enzyme dissociated into subunits that were estimated by three independent measurements to have molecular weights of about 35,000. The data thus suggest that the enzyme is composed of 6 subunits.

Several lines of evidence suggest that the subunits may be identical. Genetic studies of the "G" gene of the histidine operon which codes for the phosphoribosyltransferase have revealed no intragenic complementation (24). The existence of a single complementation unit is most consistent with the product being a single polypeptide chain. The linearity of the plot of the logarithm of the native protein divided by the subunit concentration against the logarithm of the ratio of the molecular weights of the enzyme and its subunits is shown in Fig. 5. The common slope of the plot of the logarithm of the native protein divided by the subunit concentration against the logarithm of the ratio of the molecular weights of the enzyme and its subunits suggests the enzyme is composed of 6 subunits.

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Purification and Composition Studies of Phosphoribosyl-adenosine Triphosphate: Pyrophosphate Phosphoribosyltransferase, the First Enzyme of Histidine Biosynthesis

Mary Jane Voll, Ettore Appella and Robert G. Martin


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