Evidence against a Covalent Intermediate in the Adenosine Triphosphate Phosphoribosyltransferase Reaction of Histidine Biosynthesis*

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WAYNE T. BRASHEAR AND STANLEY M. PARSONS

From the Department of Chemistry, University of California, Santa Barbara, California 93106

Histidine is synthesized in microorganisms via a pathway composed of 10 enzymatic steps which is subject to two forms of regulation (1). Feedback inhibition of the first enzyme provides a rapid response to fluctuations in the histidine pool (2, 3) while repression control of the enzyme levels provides long term control. The first enzyme of the pathway is adenosine triphosphate phosphoribosyltransferase (EC 2.4.2.17), abbreviated ATP phosphoribosyltransferase, and it catalyzes the reaction shown in Equation 1.

\[ \text{PRibPP} + \text{ATP} \rightarrow \text{PRibATP} + \text{PP}_i \]  

Equation 1

ATP phosphoribosyltransferase is a hexamer composed of 36,000 molecular weight subunits (4) which can bind histidine at an allosteric site to give inhibition of the enzymatic reaction (3). In early work on the enzyme, Martin (2) observed the exchange reactions shown in Equations 2 and 3.

\[ \text{Mg}^{2+} \text{PRibPP} + \text{PP}_i \rightarrow \text{PRibPP} + \text{Mg}^{2+} \]  

Equation 2

\[ \text{PRibATP} + [\text{14C}]\text{ATP} \rightarrow \text{PRibATP} + [\text{14C}]\text{ATP} \]  

Equation 3

The existence of exchange reactions when only one of the two substrates was present, for the reaction in either direction, suggested that an enzyme covalent intermediate was formed. This conclusion was supported when later workers (5) obtained evidence for covalent phosphoribosylation of ATP phosphoribosyltransferase using \(^{14}\)C-labeled PRibPP according to Equation 4.

\[ [\text{14C}]\text{PRibPP} + \text{enzyme} \rightarrow [\text{14C}]\text{PRibATP} + \text{PP}_i \]  

Equation 4

Inorganic pyrophosphatase was included to pull phosphoribosylation to completion, after which histidine was added to stabilize the intermediate. Up to 100% of the enzyme was observed to form the apparent intermediate.

Stabilization of an intermediate in the presence of histidine is, of course, central to the mechanism of catalysis and feedback inhibition of ATP phosphoribosyltransferase. Moreover, this phenomenon suggested an additional possibility since it is probable that ATP phosphoribosyltransferase is involved in repression of the histidine operon (6), and may also be involved in regulation of the level of histidyl-tRNA synthetase (7). The apparent phosphoribosylation of the enzyme raised the important possibility that its role in gene expression was modulated by covalent modification.

The exchange reactions and covalent intermediate are consistent with a double displacement mechanism for ATP phosphoribosyltransferase (8). However, we have recently demonstrated that this mechanism is inadequate. Since the reactant PRibPP is of \(\alpha\) stereochemistry at the pyrophosphate substitution center, the double displacement mechanism would predict that PRibATP also should be \(\alpha\). However, it is \(\beta\) (9). Also, substrate steady state initial velocity kinetic studies (2, 10) on the enzymatic reaction in both directions are consistent with a sequential mechanism (8).

The evidence accumulated against formation of a covalent intermediate under normal conditions prompted us to re-

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1 Adenosine triphosphate phosphoribosyltransferase has been incorrectly termed PR-ATP synthetase in some of the literature. The full systematic name is \(N'-(5'\)-phospho-\(\alpha\)-ribose-1-diphosphate; PRibPP, 5-phospho-\(\alpha\)-ribose-1-diphosphate; PRibATP, \(N'-(5'\)-phospho-\(\beta\)-ribose)adenosine triphosphate; PRibAMP, \(N'-(5'\)-phospho-\(\beta\)-ribose)adenosine monophosphate; BBM II, 
5-(\(\alpha\)-phospho-\(\beta\)-ribose-1-aminooformimino)-1-(\(\alpha\)-phospho-\(\beta\)-ribose)imidazole-4-carboxamide; Hepses, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; Meso, 2-(N-morpholino)ethanesulfonic acid.

5. J. Kleeman and S. M. Parsons, unpublished observations.
examine the experiments supporting this (2, 5). At the time when the previous work was performed it was not known that the enzyme can exist in a number of slowly interconvertible conformation and dissociation states (3, 11). A broad study of reaction conditions was undertaken using this information in an effort to isolate a coherent intermediate and the results are reported here.

**Materials and Methods**

ATP phosphoribosyltransferase was isolated as previously described (12) from *Salmonella typhimurium* LT2 strain TA2165 (13) or by a newer procedure (15) from strain hisE114 (14). Strain TA2165 carries the mutation his*3227 (deletes genes A, E, F, H, and I), the mutation hisO2142 which leads to derepression of the remaining genes of histidine biosynthesis including that for ATP phosphoribosyltransferase, and mutation *Chl*1031 (deletes *gal*, *Chl*, *hut*, *bioA*, and *wurB*) which eliminates a histidinase contaminant in ATP phosphoribosyltransferase isolated by the older procedure. *His*E114 carries only a point mutation in gene E of the histidine operon which leads to derepressed expression of this gene (14). The limiting LiCl concentration was achieved and frozen in liquid nitrogen for 8 to 10 hours. Some dialysis buffers also contained 50% (v/v) presence of very high Mg2+ (Table I, Experiment 21). No clear

**Results**

A large number of experiments under a wide range of conditions known to affect enzyme activity and association state were carried out in an effort to find optimal conditions for labeling of the enzyme by [14C]PRibPP. These are summarized in Table I. Some radioactivity emerged with the enzyme after gel filtration when ATP phosphoribosyltransferase and [14C]PRibPP were incubated under conditions which stabilize fully active hexameric enzyme (3). However, apparent maximal labeling of only several per cent of the subunits could be achieved and this depended upon the presence of high enzyme concentrations. Enzyme was not labeled when it was incubated with [14C]PRibPP under conditions known to inhibit the steady state enzymatic reaction (2, 3). No labeling was obtained for 0° indefinitely aggregated enzyme (Table I, Experiments 1, 14, 15, and 18), for enzyme fully inhibited by histidine (Table I, Experiment 3), or for reaction in the presence of very high Mg2+ (Table I, Experiment 21). No clear
TABLE I
Survey of labeling conditions

| Experiment no. | pH   | PH Enzyme concentration | Reaction time before histidine | Quenching histidine | Other ligands | % Labeling | MgCl₂ | PRibPP |
|---------------|------|-------------------------|--------------------------------|---------------------|--------------|------------|--------|--------|
| 1             | 7.5  | 390                     | 120                            | 0.8                 | -            | 0          | 2.5    | 2      |
| 2             | 7.5  | 380                     | 120                            | 0.8                 | -            | 0.02       | 2.5    | 2      |
| 3             | 7.5  | 450                     | 120                            | 0.8                 | -            | 0          | 2.5    | 2      |
| 4             | 7.5  | 260                     | 120                            | 0.4                 | -            | 0          | 2.5    | 2      |
| 5             | 7.5  | (300)                   | 190                            | 4                   | -            | 0          | 2.5    | 2      |
| 6             | 7.5  | (300)                   | 120                            | 10                  | -            | 0          | 2.5    | 2      |
| 7             | 7.5  | (300)                   | 600                            | 10                  | -            | 0          | 2.5    | 2      |
| 8             | 6.5  | 310                     | 120                            | 2                   | -            | 0          | 2.5    | 2      |
| 9             | 8.5  | 525                     | 120                            | 2                   | -            | 0          | 2.5    | 2      |
| 10            | 6.5  | 260                     | 1                              | 2                   | -            | 0          | 20     | 2      |
| 11            | 6.5  | 285                     | 120                            | 2                   | -            | 0.01       | 20     | 2      |
| 12            | 6.5  | 170                     | 120                            | 2                   | -            | 0.01       | 20     | 2      |
| 13            | 6.5  | (200)                   | 120                            | 20                  | -            | 0.01       | 20     | 2      |
| 14            | 7.5  | 385                     | 900                            | 2                   | -            | 0          | 20     | 76     |
| 15            | 7.5  | 610                     | 600                            | 2                   | -            | 0          | 20     | 76     |
| 16            | 7.5  | 385                     | 300                            | 2                   | -            | 0          | 20     | 76     |
| 17            | 7.5  | 330                     | 5                              | 2                   | -            | 0          | 20     | 76     |
| 18            | 7.5  | 200                     | 60                             | 4                   | -            | 0          | 20     | 76     |
| 19            | 7.5  | (900)                   | 7                              | 4                   | -            | (3)*       | 20     | 76     |
| 20            | 7.5  | 900                     | 7                              | 1                   | -            | 3.5        | 20     | 76     |
| 21            | 7.5  | (700)                   | 7                              | 4                   | -            | 0          | 100    | 92     |
| 22            | 7.5  | 340                     | 7                              | 1                   | -            | 0          | 20     | 92     |
| 23            | 7.5  | 550                     | 7                              | 1                   | -            | 3          | 20     | 92     |
| 24            | 7.5  | 420                     | 7                              | 1.1 ADP*            | 0.3          | 20         | 88     |
| 25            | 7.5  | 320                     | 7                              | 1.1 AMP*            | 0.0          | 20         | 88     |
| 26            | 7.5  | 390                     | 7                              | 0.005 ATP*          | 0.1          | 20         | 48     |
| 27            | 7.5  | 110                     | 7                              | 0.24 ATP*           | 75*          | 20         | 88     |

*a All experiments conducted at 25°C except where noted. Experiments 1 through 13 utilized enzyme isolated by a new procedure (13) from hisEll, while Experiments 14 to 27 utilized enzyme isolated by an old procedure from TA2165.

*b In the peak fraction after gel filtration. Due to inhibition by high levels of histidine, the values in parentheses are estimated.

c Due to uncertain enzyme concentration, the value is estimated.

d Enzyme preincubated 50 min with 0.8 mM L-histidine before reaction.

e Enzyme preincubated 120 min at 0°C before reaction at 0°C.

f Reacted at 25°C immediately after warming from 0°C.

*g Reaction solution contained 50% v/v glycerol.

h Enzyme preincubated 50 min with 0.1 mM L-histidine before reaction.

i Tris-Mes buffer system.

j Tris-HCl buffer system.

m Added 7 s after addition of histidine.

1 Extensive labeling also could be obtained in the presence of high levels of ATP under other experimental conditions, as detailed in the text.

correlation of labeling with variation in pH, reaction time, concentration of trapping histidine, presence of glycerol, or PRibPP concentrations was observed.

Since it has been proposed (18) that some chemical transformations in enzymes are dependent upon the presence of a second substrate molecule, even though it does not participate in the reaction under study, it was possible that additional ligands might induce labeling. The ATP phosphoribosyltransferase substrate analogs AMP and ADP were tried. Both bind to the enzyme but are not converted to products (2, 19). No labeling was found after reaction in the presence of AMP and little was found after the presence of ADP (Table I, Experiments 24 and 25). Thus “substrate synergism” could not be demonstrated using AMP and ADP.

However, when excess ATP was included, a very high level of apparent labeling was found (Fig. 1). Labeling did not depend upon order of addition of ATP and [14C]PRibPP (not shown). This extensive labeling was completely blocked if the enzyme was preincubated for 50 min in the presence of histidine (Fig. 2), but only slightly blocked if it was preincubated for only 7 s in the presence of histidine (Fig. 3). This latter behavior is similar to the lag in histidine inhibition seen for enzyme which has not been exposed recently to histidine (3, 18). The high level of labeling in the presence of ATP thus responds to histidine inhibition in the same manner as steady state formation of products. Also, a trace level of ATP added 7 s after addition of histidine gave little labeling (Table I, Experiment 26). Thus, stoichiometric and not catalytic levels of ATP are required for extensive labeling. Finally, it was found that histidine was not required at all for labeling since reaction under the conditions of Fig. 1 in the complete absence of histidine led to 75% labeled enzyme (Table I, Experiment 27).

Enzyme which had been highly labeled was isolated by gel filtration as in Fig. 1, denatured with urea, and gel-filtered in urea to determine whether radioactivity was still bound to the protein. Fig. 4 shows that it was not. Also, the labeled native
FIG. 1 (left). Labeling of ATP phosphoribosyltransferase by substrates. The enzyme (200 μl, about 13-nmol subunits) in the standard buffer of 0.1 M NaCl, 0.01 M Tris, 0.5 mM EDTA, 20 mM MgCl₂, 10 mM dithiothreitol, and 50% (v/v) glycerol, adjusted to pH 7.5 with solid Hepes was incubated at 25° for 50 min with 0.4 unit of pyrophosphatase. [14C]PRibPP (6 μl, 10 nmol containing 22,000 cpm) was added, followed 7 s later by ATP (5 μl, 125 nmol). After 7 s of incubation histidine (5 μl, 500 nmol) was added to give 2.3 mM histidine and the solution immediately gel-filtered. Aliquots (500 μl) of each fraction were counted for radioactivity and 5-μl aliquots were assayed for enzyme activity. Approximately 70% labeling of subunits was present in the peak tube at Number 7. Similar results were obtained in the absence of glycerol.

FIG. 2 (left center). Effect of prolonged incubation with histidine on labeling. The enzyme was treated as in Fig. 1 except that it was made up to 2 mM histidine 50 min before addition of [14C]PRibPP and ATP. No labeling occurred. Order of addition of [14C]PRibPP and ATP did not matter.

FIG. 3 (right center). Effect of brief incubation with histidine on labeling. The enzyme was treated as in Fig. 1 except that it was made 2.5 mM in histidine 7 s after addition of [14C]PRibPP and 7 s before addition of ATP. Approximately 68% labeling was found.

FIG. 4 (right). Urea-denatured [14C]-labeled ATP phosphoribosyltransferase. Enzyme (0.5 ml) which had been highly labeled and isolated as in Fig. 1 was saturated with 700 mg of urea and incubated at 25° for 30 min before gel filtration in urea. No radioactivity remained bound to the enzyme as detected by its absorbance at 280 nm. Several alternative denaturation procedures also gave no labeling of the enzyme.

These results made it probable that the radioactive material bound to the enzyme was the product [14C]PRibATP. This was tested by incubating the enzyme in the presence of labeled PRibPP, histidine, and ATP under the conditions of Fig. 1, with the subsequent addition of a partially purified preparation of the second and third enzymes of the histidine pathway genetically designated E and I. These enzymes convert PRibATP to PRibAMP and BBM II (1). Gel filtration of the reaction product showed 90% diminished labeling of ATP phosphoribosyltransferase compared to the control (not shown). Also, a heavily labeled enzyme solution similar to that gel-filtered in Fig. 1 was analyzed by paper electrophoresis. A predominant new peak of radioactivity having a similar electrophoretic mobility to authentic PRibATP was present along with excess [14C]PRibPP and a diffuse peak near the origin probably arising from [14C]PRibATP bound to the enzyme (Fig. 5). Thus the radioactive label behaved like PRibATP.

Since extensive labeling of ATP phosphoribosyltransferase by [14C]PRibPP could not be demonstrated under any set of conditions tried unless ATP was added, it seemed probable that the low level of labeling observed with [14C]PRibPP was due to contaminating ATP. To test this, an enzyme solution reacted with [14C]PRibPP was treated with E and I enzymes. Gel filtration of the reaction product showed no labeling of ATP phosphoribosyltransferase (Fig. 6B), whereas a control without E and I enzymes showed 0.2% labeling (Fig. 6A). When the labeled PRibPP was pretreated with hexokinase and glucose before incubation with ATP phosphoribosyltransferase...
ase, labeling of ATP phosphoribosyltransferase again was much reduced (Fig. 6C). Paper electrophoresis of the labeled PRibPP after treatment with hexokinase and glucose demonstrated that most of the \[^{14}\text{C}\]PRibPP remained intact (not shown) and thus was available for labeling of the enzyme. The low level of labeling also was eliminated if inorganic pyrophosphate was included in the absence of pyrophosphatase (not shown). This was considered by other workers (5) to be compatible with reaction of an intermediate, but this result also is compatible with conversion of products to reactants. Thus these experiments suggest that the low level of labeling of ATP phosphoribosyltransferase by \[^{14}\text{C}\]PRibPP is due to formation of \[^{14}\text{C}\]PRibATP from contaminating ATP.

**DISCUSSION**

Extensive attempts to produce labeling of adenosine triphosphate phosphoribosyltransferase by \[^{14}\text{C}\]PRibPP in the supposed absence of ATP resulted in only trace amounts of radioactivity bound to the enzyme. This was despite the fact that the \[^{14}\text{C}\]PRibPP utilized in this study was of 50 times greater specific activity than that used previously by other workers who achieved labeling while enzyme concentrations were approximately the same (5). The experimental conditions explored included those in which the enzyme exists in two forms of the hexamer and a high molecular weight aggregated form (11). Enzyme isolated using two different procedures (12, 13) gave the same results. A high level of labeling could not be reproduced under any environmental variation.

Heavy labeling could be obtained only in the presence of high concentrations of ATP. This labeling was shown to be capable of transformation to \[^{14}\text{C}\]PRibATP since it largely was eliminated by other enzymes of the histidine pathway which act on PRibATP, and most of the label possessed the electrophoretic mobility of PRibATP. In addition, no label remained bound to the enzyme after urea denaturation, thus demonstrating that the label bound to native enzyme was not linked by a stable covalent bond. There was no evidence that the radioactivity bound to the native enzyme was some form of \[^{14}\text{C}\]PRibPP which was covalently bound only under conditions of “substrate synergism.” Neither of the substrate analogs AMP or ADP gave heavy labeling. Complexation by EDTA of the magnesium in a solution containing enzyme highly labeled in the presence of ATP gave no labeling after urea denaturation. If a covalent phosphoribosylated enzyme were formed in the presence of magnesium-ATP, the covalent phosphoribosyl group might have been trapped in a form which was unable to transfer from the enzyme to ATP in the absence of magnesium. Thus no evidence could be obtained that the radioactivity in extensively labeled enzyme was covalently bonded either before or after denaturation.

The dissociation constant of PRibATP recently has been determined to be 1.8 \(\mu\text{M}\) under assay conditions.\(^8\) Since the typical enzyme concentration utilized in these studies is larger than this dissociation constant (1000 units of ATP phosphoribosyltransferase/ml equals \(1.2 \times 10^{-3} \text{ M}\) subunits), PRibATP would travel with the enzyme during gel filtration on a short column of Sephadex. Thus the heavy labeling of the enzyme in the presence of ATP probably was due to \[^{14}\text{C}\]PRibATP tightly but noncovalently bound to the enzyme.

Trace labeling which could be obtained in the supposed absence of ATP was blocked under conditions known to inhibit over-all steady state activity of the enzyme. The trace labeling was reduced greatly when the \[^{14}\text{C}\]PRibPP was pretreated with hexokinase and glucose, which would convert any ATP present to ADP, or when the labeled enzyme was treated with enzymes acting on PRibATP. Therefore the trace labeling probably also was due to \[^{14}\text{C}\]PRibATP arising from contaminating ATP in the \[^{14}\text{C}\]PRibPP which we prepared.

Thus we have concluded from the results presented here that labeling of the enzyme occurs only by the noncovalent binding of the PRibATP product of the enzymatic reaction. How can we explain the results of previous workers (5) who observed binding of PRibPP alone? The most likely explanation is that the PRibPP preparation used in the previous study was

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**Fig. 6.** Labeling of ATP phosphoribosyltransferase in the absence of added ATP. Graph A (0.2% labeling) is a typical labeling pattern obtained when relatively high concentrations of enzyme are treated with \[^{14}\text{C}\]PRibPP in the absence of ATP. These experiments were conducted with enzyme in standard buffer without glycerol at pH 7.5. Enzyme (500 \(\mu\text{l}\), about 32-nmol subunits) was incubated 60 min at 25\(^\circ\)C with 0.5 unit of pyrophosphatase, followed by the addition of \[^{14}\text{C}\]PRibPP (14 \(\mu\text{l}\), 40 nmol containing 45,000 cpm) and incubation for 7 s, followed by the addition of histidine (4 \(\mu\text{l}\), 400 nmol) to give 0.8 \(\text{mM}\) histidine. To 250 \(\mu\text{l}\) of this solution 25 \(\mu\text{l}\) of partially pure enzyme (500 \(\mu\text{l}\), about 32-nmol subunits) was incubated 60 min at 25\(^\circ\)C with 0.5 unit of pyrophosphatase, followed by the addition of \[^{14}\text{C}\]PRibPP (14 \(\mu\text{l}\), 40 nmol containing 45,000 cpm) and incubation for 7 s, followed by the addition of histidine (4 \(\mu\text{l}\), 400 nmol) to give 0.8 \(\text{mM}\) histidine. To 250 \(\mu\text{l}\) of this solution 25 \(\mu\text{l}\) of partially pure E and I enzymes was added. The solution was incubated 30 min at 25\(^\circ\)C, and gel-filtered in the standard buffer plus 1 \(\text{mM}\) histidine. This gave Graph B. The remaining solution, untreated by E and I enzymes, then was immediately gel-filtered and gave Graph A. \[^{14}\text{C}\]PRibPP treated with hexokinase and glucose as detailed under “Materials and Methods” was used for labeling 200 \(\mu\text{l}\) of enzyme using the procedure of Graph A, except that the product was gel-filtered immediately after quenching with histidine. The result is shown in Graph C. Decreased labeling seen in B and C suggests that the labeling in A is due to PRibATP.
contaminated with ATP, that this contaminant allowed conversion of labeled PRibPP to labeled PRibATP, and that binding of this PRibATP was erroneously attributed to an intermediate. Their synthesis of PRibPP was accomplished enzymatically with ATP using a procedure (20) which involved much less care than our synthesis, and undoubtedly greater contamination by ATP. Also, the $^{32}$PPi exchange into PRibPP in the presence of the enzyme (Equation 2) which was observed by Martin (2) could have been due to the unsuspected presence of ATP since commercial PRibPP also is synthesized enzymatically.\(^3\) With both substrates present, both products would be formed and back-reaction would give PRib$[^{32}$P]PP. Thus, exchange could occur in this situation without the intermediacy of a phosphoribosylated enzyme. The $[^{14}$C]ATP exchange into PRibATP (Equation 3), also observed by Martin, similarly could have been due to the presence of pyrophosphate which easily could arise from breakdown of PRibATP or ATP. Thus results of previous workers can be reasonably explained without invoking a covalent intermediate.

The negative evidence presented here cannot rigorously rule out the existence of a phosphoribosylated enzyme bound to ATP in the native state which can react to give PRibATP under the conditions used to test for PRibATP. However, there is no reason to invoke this special case. Thus there is currently no good evidence for a covalent intermediate in the ATP phosphoribosyltransferase reaction.

Tight binding of PRibATP also could cause the apparent differences between pure enzyme and enzyme in crude bacterial extracts reported previously (13). Bound PRibATP from a crude extract probably would stabilize the hexameric form of the enzyme (11). This would make crude enzyme resistant towards cold inactivation and would change the precipitation behavior of the enzyme in Ouchterlony immunodiffusion tests as was observed.

Many of the results presented here are essentially single turnover experiments since the concentration of enzyme subunits was greater than or approximately equal to the $[^{14}$C]PRibPP available in most cases. Enzyme preincubated in the presence of histidine for an extended time produced no bound $[^{14}$C]PRibATP in the presence of $[^{14}$C]PRibPP and added ATP. Thus histidine apparently inhibits ATP phosphoribosyltransferase at the step involving reaction between PRibPP and ATP, or at some earlier step. Histidine inhibition due to slowed product release from the enzyme clearly is excluded as a dominant mechanism for inhibition of the steady state reaction since not even enzyme levels of product are formed under these conditions. Also, histidine was demonstrated to be not an effective inhibitor of PRibATP formation within 7 s after being mixed with the enzyme. This result confirms previous observations that enzyme which has not been recently exposed to histidine requires several minutes before steady state activity is completely inhibited upon addition of histidine (3, 19).

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*Personal communications from P-L Biochemicals and Sigma Chemical Co. ATP contamination appears to be very low in modern commercial preparations of PRibPP.*
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W T Brashear and S M Parsons

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