A PHOSPHORIBOSYL-ENZYME COVALENT INTERMEDIATE IN THE FIRST ENZYME OF HISTIDINE BIOSYNTHESIS

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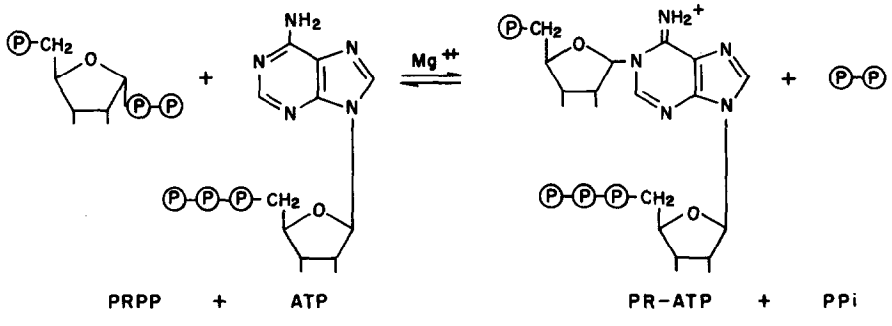
SUMMARY: Evidence is presented that the mechanism of phosphoribosyl-adenosine triphosphate: pyrophosphate phosphoribosyltransferase (the first enzyme of histidine biosynthesis) proceeds through a covalent phosphoribosyl-enzyme intermediate. The intermediate has been demonstrated after incubating the enzyme with ^{14}C-PRPP under both "native" and denaturing conditions. The intermediate also forms from the reverse direction as demonstrated by an initial burst phenomenon when the enzyme is mixed with its product, PR-ATP. Thus, exchange data coupled with specificity requirements appears to provide sound evidence for a covalent enzyme-substrate intermediate.

The first enzyme of the histidine biosynthetic pathway of *Salmonella typhimurium* is of particular interest because it plays an important role in regulation of the pathway (1-3) and because its mechanism of action suggests a sugar-protein intermediate (4). Martin found that the enzyme (phosphoribosyl-adenosine triphosphate: pyrophosphate phosphoribosyltransferase (E.C. 4.2.1C) which catalyzes the overall reaction shown in Equation 1 also catalyzes an



(1)

[1] Abbreviations: PRPP, 5-phosphoribosyl-1-pyrophosphate; PR-ATP, N-1-(5'-phosphoribosyl)-adenosine triphosphate.

exchange between ^{32}P-pyrophosphate and PRPP[1] in the absence of ATP and between ^{14}C-ATP and PR-ATP in the absence of PRPP (4). The first postulation of a covalent intermediate by such exchange reactions was the classic study of Doudoroff, Barker and Hassid in the case of sucrose phosphorylase (5). Although exchange per se is not proof of an enzyme-substrate intermediate (6), it has been pointed out that exchange data coupled with specificity requirements should be evident for a covalent intermediate (7,8). The prediction of a covalent intermediate has been verified in the case of sucrose phosphorylase by the studies of Abeles and Voet (9,10). The same exchange-specificity criteria would therefore predict that a covalent intermediate should be found in the phosphoribosyl-adenosine triphosphate: pyrophosphate phosphoribosyltransferase enzyme. In this paper we report evidence for such an intermediate.

MATERIALS AND METHODS

Enzyme Assay. Phosphoribosyl-adenosine triphosphate: pyrophosphate phosphoribosyltransferase activity was assayed by the methods of Ames *et al.* (1) as modified by Voll, Apella and Martin (11).

Enzyme. Phosphoribosyl-adenosine triphosphate: pyrophosphate phosphoribosyltransferase was purified from *Salmonella typhimurium* strain E11 by a modification of the procedure of Voll *et al.* (11).

Sephadex G-25 Chromatography. G-25 Sephadex chromatography was performed in three different buffer systems: Buffer A - 0.01 M Tris-HCl, 0.10 M NaCl, 0.5 mM EDTA, 1.0 mM dithiothreitol, pH 7.5; Buffer B - Buffer A plus 0.4 mM L-histidine; and Buffer C - 0.01 M potassium phosphate and 5 M urea, pH 7.0.

^{14}C-PRPP. ^{14}C-PRPP was synthesized enzymatically from ^{14}C-ribose-5-phosphate (Schwarz Bio-Research, Inc.) and ATP using PRPP synthetase from *Salmonella typhimurium* (12) and purified according to the procedure of Kornberg and Khorana (13).

N-1-(5'-phosphoribosyl)-ATP. PR-ATP was prepared enzymatically by a modification of the procedure reported by Smith and Ames (14).

RESULTS

In an attempt to demonstrate the existence of an intermediate the enzyme was incubated with ^{14}C-PRPP and magnesium chloride. Yeast inorganic pyrophosphatase was added to displace the equilibrium in favor of formation of the

$$^{14}\text{C-PRPP} + \text{E} \xrightarrow{Mg^{++}} {}^{14}\text{C-PR-E} + \text{PP}_i \xrightarrow{\text{PP}_i\text{ase}} 2\text{P}_i \qquad (2)$$

intermediate as shown in Equation 2. Urea was then added until the concentration reached 6 M where the enzyme is known to be inactive and dissociated (11). This solution was placed on a G-25 Sephadex column equilibrated with 5 M urea. The results shown in Figure 1 present strong evidence for a covalent inter-

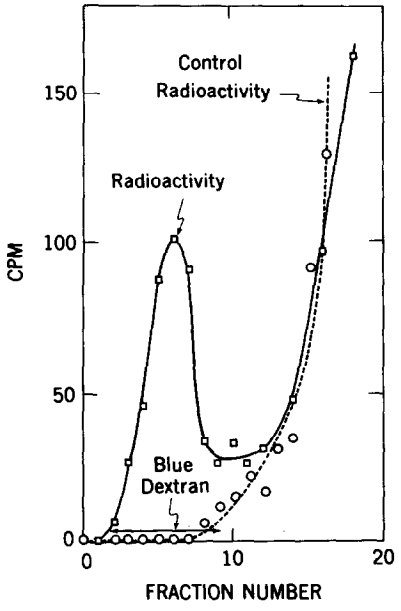


Figure 1. Resolution of ^{14}C-phosphoribosyl-enzyme intermediate from ^{14}C-PRPP using G-25 Sephadex chromatography under denaturing conditions. ^{14}C-Phosphoribosyl-enzyme complex was produced in a reaction mixture containing 1.4 mg protein in 0.45 ml buffer A, 4.5 µmoles $MgCl_2$, 0.5 µmoles ^{14}C-PRPP and 1.8 units of yeast inorganic pyrophosphatase (Worthington). After 30 seconds at room temperature, solid urea was added to make the solution 6 M and the mixture was applied to a G-25 Sephadex column (1.5 x 25 cm) equilibrated with buffer C and calibrated with Blue Dextran. The control consisted of enzyme already in 6 M urea before the ^{14}C-PRPP was added. Radioactivity, □-□. control radioactivity, 0-0.

mediate. A radioactive peak was found in the breakthrough volume in the above experiment whereas the peak was absent in a control experiment in which ^{14}C-PRPP was added to inactive enzyme already in 6 M urea.

Since the enzyme does not utilize water as a normal acceptor, it seemed possible that the intermediate might be isolated without denaturation and therefore an experiment was initiated in which the enzyme was chromatographed without denaturation after incubation with ^{14}C-PRPP. Again it was noted (cf. Figure 2) that a peak of radioactivity appeared in the breakthrough volume which in this experiment also coincided with the enzymatic activity. Although it could be argued that such a peak might arise from non-covalent binding,

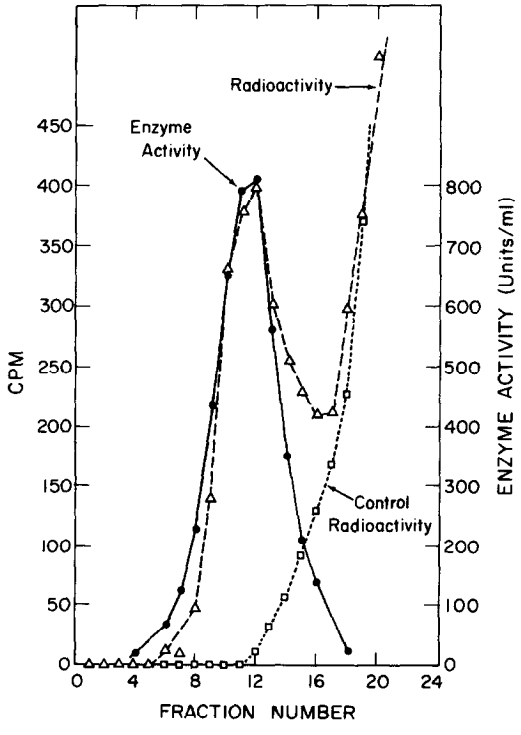


Figure 2. Resolution of ^{14}C-phosphoribosyl-enzyme intermediate from ^{14}C-PRPP using G-25 Sephadex chromatography under non-denaturing conditions. Phosphoribosyl-enzyme complex was produced in a reaction mixture containing 1 ml of enzyme in buffer A, 0.15 µmoles $MgCl_2$, 0.5 µmoles ^{14}C-PRPP and 2.7 units of yeast inorganic pyrophosphatase. After two minutes at room temperature, 50 µl of a 15 mM L-histidine solution at pH 8.5 was added. The reaction mixture was applied to a G-25 Sephadex column (1.5 x 30 cm) equilibrated with buffer B at 4°C. The control did not contain enzyme. 0-0, enzyme activity; Δ-Δ, radioactivity, □-□, control radioactivity.

this alternative was eliminated by the finding that the peak was absent when either pyrophosphate or ATP was added to the labeled enzyme. The absence of a peak after such treatment is consistent with removal of a covalently bound ^{14}C-phosphoribosyl moiety from the enzyme by reaction with these substrates.

Further evidence for the existence of a phosphoribosyl-enzyme intermediate was obtained by difference specta at 290 mμ when PR-ATP was added to the enzyme. The high extinction at 290 mμ is associated with the N-1'-phosphoribosyl-glycosidic bond of PR-ATP (1). As shown in Figure 3, a loss of absorption at 290 mμ was observed which was proportional to the enzyme concentration. This is consistent with the breaking of the N-1'-phosphoribosyl-glycosidic bond of PR-ATP and the formation of a phosphoribosyl-enzyme. It is characteristic of an initial burst phenomenon such as that observed for chymotrypsin in the presence of various substrates (15-17).

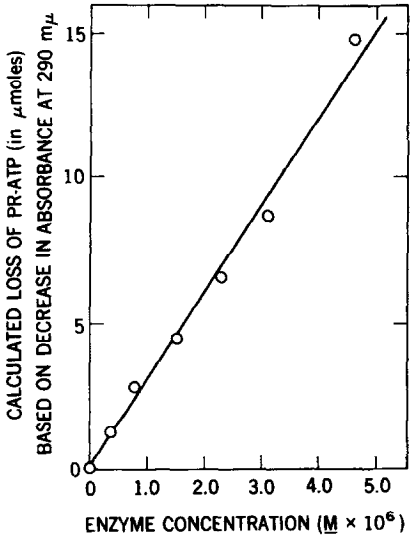


Figure 3. Formation of phosphoribosyl-enzyme intermediate from PR-ATP. Difference spectra at 290 mμ obtained using tandom cells. Loss of PR-ATP was calculated from the observed decrease in absorption at 290 mμ from the difference in the extinction coefficients between ATP and PR-ATP at 290 mμ, pH 8.5 (18). PR-ATP at 6.86 x 10^{-4} M was made up in 1 M Tris buffer, pH 8.5. Enzyme at 9.2 x 10^{-6} M was diluted in 1 M Tris, pH 8.5, to the desired concentration. Both the PR-ATP solution and the enzyme solution were incubated with 4.5 units of yeast inorganic pyrophosphatase for 20 minutes at room temperature to eliminate traces of pyrophosphate.

Finally, stoichiometries up to 6.0 moles of phosphoribosyl groups bound per mole of enzyme have been calculated from the specific activity of the ^{14}C-PRPP. Since the enzyme has been found to consist of 6 identical subunits (11), the stoichiometry is consistent with the reported subunit structure.

The finding of a covalent intermediate in this enzyme is interesting since it provides a tool for investigating the role of the allosteric effector, and the nature of the covalent intermediate. It also adds further support for the exchange-specificity criterion as evidence of a covalent intermediate. The essence of this argument is shown schematically in Figure 4. If B-X is the donor and Y the acceptor in an enzymatic transfer reaction, we can see that exchanges of the type B-X with X* and B-Y with Y* can only occur by a single displacement mechanism when X* can occupy the site normally occupied by Y or Y* can occupy the site normally occupied by X. If the specificity pattern excludes this possibility (ATP cannot occupy the PP site and *vice versa*), then positive

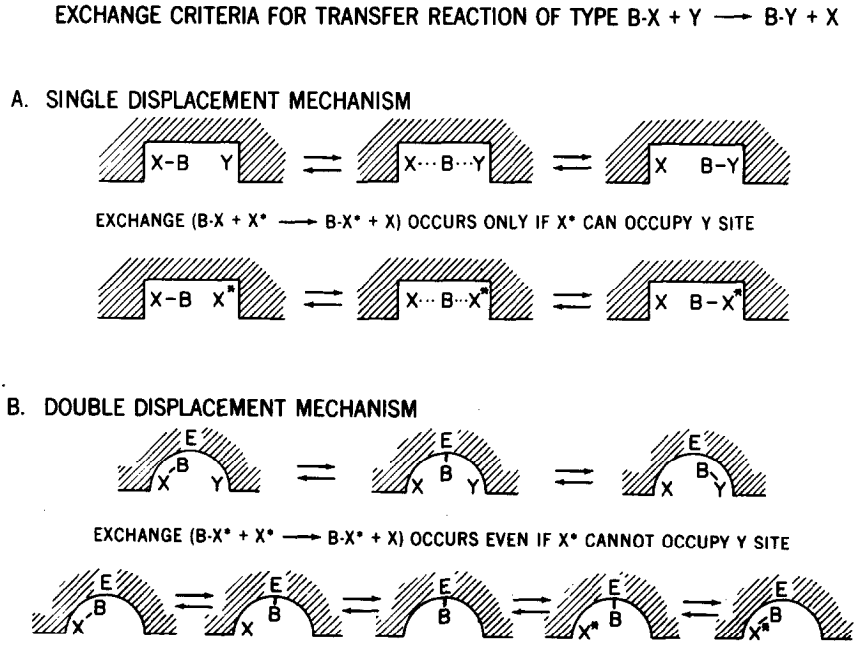


Figure 4. Illustration of the exchange-specificity criterion (see text).

exchange experiments indicate a double displacement mechanism. When this criterion was formulated on theoretical grounds, no covalent intermediates had been isolated. Today it appears that whenever the exchange-specificity criterion have been met that an appropriate search has yielded the expected covalent intermediate. It would therefore appear that the exchange-specificity criterion is sound as an indicator of an intermediate. It remains to be seen whether the prediction of stereochemistry can be verified in this case as it has been for sucrose phosphorylase.

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