MECHANISM OF PHOSPHORIBOSYLPYROPHOSPHATE SYNTHETASE: EVIDENCE FOR AN ENZYME-PYROPHOSPHATE INTERMEDIATE

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The enzyme 5-phosphoribosyl-α-1-pyrophosphate (PRPP) synthetase (ATP:D-ribose-5-phosphate pyrophosphotransferase, E. C. 2.7.6.1) catalyzes the first step of a highly branched biosynthetic pathway which leads ultimately to the synthesis of purines, pyrimidines, tryptophan, histidine, and pyridine nucleotides. Feedback inhibition of PRPP synthetase from Salmonella typhimurium and Escherichia coli by the various end products of PRPP metabolism has been reported (Switzer, 1967; Atkinson and Fall, 1967). As part of a study of the details of the regulation and mechanism of action of PRPP synthetase from S. typhimurium, it was found that the purified enzyme catalyzes an exchange between ^{14}C-AMP and ATP in the absence of ribose compounds and an exchange between ^{14}C-ribose-5-phosphate and PRPP in the absence of adenine nucleotides (R.L. Switzer, unpublished experiments). These results suggested the participation of an enzyme-pyrophosphate intermediate in the PRPP synthetase reaction. This communication presents direct evidence for the formation of such an intermediate and its function in the overall enzyme reaction.

METHODS

Preparation of PRPP synthetase. PRPP synthetase was purified from S. typhimurium LT-2 grown on the E medium of Vogel and Bonner (1956) with 0.5% glucose added. The enzyme was purified about 450 fold by procedures, to be described in detail elsewhere, which include sonic or French pressure cell disruption of the bacteria, streptomycin treatment, heating at 55°, and repeated ammonium sulfate and acid

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fractionations. The enzyme appears to be nearly homogeneous as judged by polyacrylamide disc gel electrophoresis at pH 8.7.

Assay of PRPP synthetase. PRPP synthetase was assayed by using a modification of the two step orotate removal assay of Kornberg et al. (1955). In the first step, the reaction mixture contained in 1.0 ml: 3 mM ATP, 5 mM ribose-5-phosphate, 2 mM $MnCl_2$, 0.1 M potassium phosphate buffer, pH 7.5, and sufficient enzyme to catalyze synthesis of 0.02 to 0.20 μmole of PRPP in 10 min at 37°. The reactions were terminated by heating at 100° for 60 sec; the tubes were then immediately cooled by placing in an ice bath. The second step, in which conversion of PRPP accumulated in the first step to a mixture of orotidylic acid and uridylic acid is followed spectrophotometrically, was performed essentially as described by Kornberg et al. (1955).

Radiochemicals. Radioactivity was determined by liquid scintillation counting in Bray's solution (Bray, 1960). Radiochemical purity of isotopically labelled ATP was determined by thin layer chromatography followed by radioautography and scintillation counting of areas cut from the plastic sheets. Adenosine triphosphate-γ-^{32}P, purchased from International Chemical and Nuclear Corporation, was about 92% radiochemically pure. The only radioactive impurity identified was 5% inorganic phosphate. Adenosine triphosphate, uniformly labelled with ^{14}C (New England Nuclear Corporation) was estimated to be about 97% radiochemically pure. Approximately 2% of the radioactivity was present as ADP and traces as adenosine.

RESULTS

Formation of a complex between PRPP synthetase and all or part of the ATP molecule was detected by mixing the enzyme with radioactive ATP in the presence of 50 mM potassium phosphate buffer, pH 7.5 and 10 mM $MgCl_2$ and in the absence of the pyrophosphate acceptor, ribose-5-phosphate. The enzyme was then separated from free ATP and other small molecules by chromatography at room temperature on Sephadex G-25. The experiment in Fig. 1 (upper frame) demonstrates that radioactivity from ATP-γ-^{32}P is tightly bound to the protein fraction. An experiment with uniformly labelled ^{14}C-ATP (Fig. 1, lower frame) shows that no more than 3 to 4% of the ^{32}P bound in the first experiment could have originated from binding of intact ATP; in other words, the adenosine portion of the ATP molecule is not bound to the enzyme.

An unusual property of PRPP synthetase from S. typhimurium is an absolute and specific requirement for high levels of inorganic phosphate for activity; removal

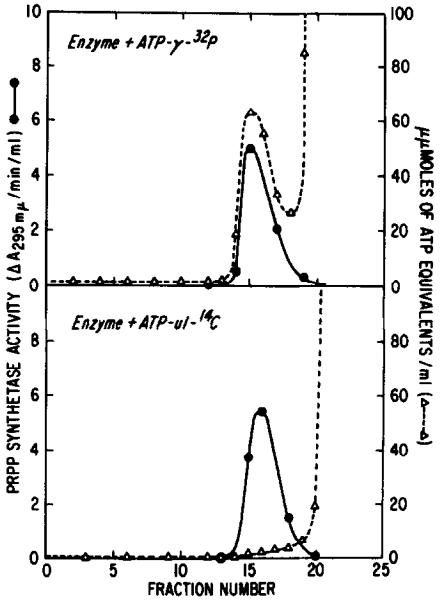

Figure 1. Labelling of PRPP synthetase by radioactive ATP. PRPP synthetase (0.2 mg, 22.5 units/mg) was incubated with 0.25 μmole radioactive ATP, 5 μmoles $MgCl_2$, and 25 μmoles potassium phosphate, pH 7.5, in a final volume of 0.5 ml. After 60 sec of reaction at 37°, the sample was chromatographed on a 1.2 cm (dia.) x 30 cm column of Sephadex G-25 (coarse bead form), eluting with 0.1 M potassium phosphate, pH 7.5. Fractions of one ml each were analyzed for radioactivity (Δ---Δ, expressed as ATP equivalents) and for PRPP synthetase activity (●—●). Upper frame: ATP-γ-^{32}P, 10.4 X 10^6 cpm/μmole, was used. Lower frame: uniformly labelled ^{14}C-ATP, 33.1 X 10^6 cpm/μmole, was added.

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of phosphate by overnight dialysis against 10 mM triethanolamine chloride buffer, pH 7.5 at 4°, brings about a total and irreversible loss of ability to catalyze the overall synthetic reaction and both of the exchange reactions (Switzer, 1968, and unpublished experiments). Incubation of such a dialyzed, inactive enzyme with ATP-γ-^{32}P (3 X 10^8 cpm/μmole) and $MgCl_2$, followed by chromatography using conditions similar to those described in Fig. 2, showed that labelling of the protein was reduced to less than 5% of a control with undialyzed enzyme. This result shows that under these conditions catalytic activity of PRPP synthetase is required for labelling of the protein fraction. Since dialysis is a rather mild and selective means of inactivating the enzyme, this experiment makes it unlikely that the observed labelling of the undialyzed enzyme is a consequence of phosphorylation of an impurity

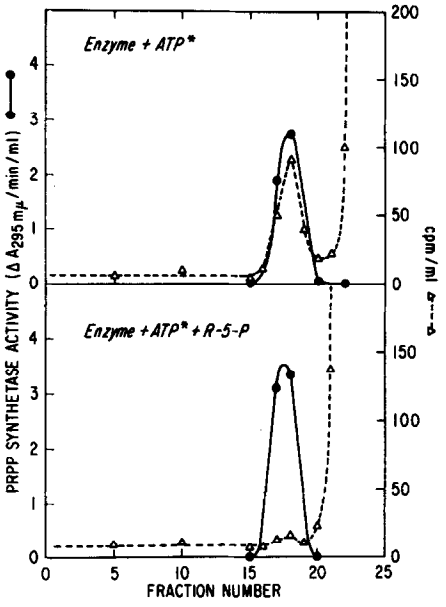

Figure 2. Effect of ribose-5-phosphate on labelling of PRPP synthetase by ATP-γ-^{32}P. The conditions were as in Fig. 1, except that a 1.5 cm (dia.) X 50 cm Sephadex G-25 column was used and two ml fractions were collected. The specific activity of the ATP-γ-^{32}P was 5.78 X 10^6 cpm/μmole. Upper frame: control without ribose-5-phosphate. Lower frame: 2.5 μmoles of ribose-5-phosphate was added to the reaction mixture before 60 sec incubation and chromatography.

in the enzyme preparation or that phosphorylation occurs at a site which is entirely distinct from the catalytic site.

Formation of labelled enzyme from ATP-γ-^{32}P is largely abolished when an excess of ribose-5-phosphate is included in the incubation mixture (Fig. 2, lower frame); a control without ribose-5-phosphate, shown in the upper frame of Fig. 2, shows the expected labelling of the enzyme. This result suggests that the phosphorylated form of the enzyme is an enzyme-pyrophosphate compound whose accumulation is prevented by the presence of the pyrophosphate acceptor, as would be expected if it functions as an intermediate in the PRPP synthetase reaction.

DISCUSSION

Although the experiments in this communication do not conclusively establish that the phosphorylated form of PRPP synthetase described is an enzyme-pyrophosphate

(as opposed to some other phosphorylated form), such a structure is suggested by the chemistry of the forward and reverse reactions catalyzed by the enzyme, i.e.,

$$\text{ATP} + \text{ribose-5-phosphate} \rightleftharpoons \text{PRPP} + \text{AMP},$$

and the exchange reactions referred to in the introduction. Final proof of the structure of the phosphorylated derivative and demonstration of its ability to serve as an intermediate in the PRPP synthetase reaction depends on studies of the chemical properties of the isolated intermediate. A few preliminary attempts to demonstrate the ability of the ^{32}P-labelled enzyme obtained from Sephadex chromatography to react with AMP or ribose-5-phosphate to form ATP or PRPP, respectively, have been unsuccessful. Although this failure may be due to purely technical difficulties, it is also possible that the phosphorylated enzyme undergoes alterations in structure during isolation under the conditions described. Systematic evaluation of the effects of temperature, phosphate and divalent cation concentration, and pH on the isolation of the labelled enzyme are in progress. It will be necessary to devise means of isolating preparations of phosphorylated enzyme which are capable of forming ATP and PRPP before definitive studies on the structure of the suspected enzyme-pyrophosphate can be performed.

There are numerous examples of enzyme-phosphate compounds which are thought to serve as intermediates in enzyme reactions (for some examples, see: Najjar and Pullman, 1954; Schwartz and Lipmann, 1961; Ramaley et al., 1967). On the other hand, evidence for the formation of enzyme-pyrophosphate intermediates has been obtained in only a few instances. Two different, but related, phosphoenolpyruvate synthetases from E. coli (Cooper and Kornberg, 1967a, 1967b) and from propionibacteria (Evans and Wood, 1968) have been reported in which it is proposed that the enzyme-pyrophosphate initially formed is cleaved to form an enzyme-phosphate and phosphate, in one case, and an enzyme-phosphate and pyrophosphate, in the other case. It seems quite possible that these enzymes will be found to possess common chemical and mechanistic features. In particular, it will be of interest to compare the chemistry of the protein-pyrophosphate linkages in these enzymes. It is hoped that, in addition to contributing to our knowledge of these unusual pyrophosphate transfer reactions, further studies will lead to an intimate understanding of the mechanism of feed-back inhibition of PRPP synthetase.

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