The Nonenzymatic Preparation in Solution of N-(5'-Phosphoribosyl)anthranilic Acid, an Intermediate in Tryptophan Biosynthesis*

(Received for publication, August 9, 1968)

THOMAS E. CREIGHTON

From the Department of Biology, Yale University, New Haven, Connecticut 06520

SUMMARY

A very rapid and convenient method of preparing in solution N-(5'-phosphoribosyl)anthranilic acid, an intermediate in tryptophan biosynthesis, is described. Although the intermediate is very unstable and has not been purified, it is suitable for use in enzymatic analysis.

Yanofsky (1) originally proposed N-(5'-phosphoribosyl)anthranilic acid as the first intermediate after anthranilic acid in tryptophan biosynthesis in microorganisms. Evidence was subsequently found which suggested that this compound is formed enzymatically from anthranilic acid and ribosylpyrophosphate 5-phosphate (2, 3). Although the dephosphorylated form of PRA, N-ribosylanthranilic acid, has been synthesized and characterized (4), repeated attempts to synthesize PRA have been unsuccessful (2, 5, 6). The elusiveness of this intermediate has been ascribed to its lability. For example, tryptophan-requiring mutants of microorganisms which would be expected to accumulate PRA or its dephosphorylated form are found to accumulate primarily anthranilic acid, which is presumably a breakdown product. Synthetic N-ribosylanthranilic acid has a half-life of approximately 30 min at pH 7.0 and 37° (5). It is the purpose of this communication to describe an extremely simple method of preparing PRA in solution which makes it available as a substrate for enzymatic studies.

EXPERIMENTAL PROCEDURE

Materials—Anthranilic acid was recrystallized once from hot water. A 1.0 M stock solution in 95% ethanol was stored in the dark at 4°. A 1.0 M stock solution of the sodium salt of α-ribose-5-P (Sigma) was kept at −15°. Both of these stock solutions could be stored over a period of several weeks with no apparent change in their properties relevant to this investigation. All other materials were obtained commercially and used without further purification.

Bacterial Strains—Escherichia coli mutant strains trp C9830 and trp C9941 were kindly supplied by Dr. C. Yanofsky. Enzymes—The normal E. coli enzyme with both PRA isomerase and InGP synthetase activities was purified to apparent homogeneity as previously described (7). The mutationally altered forms of this enzyme produced by strains trp C9830 and trp C9941 could be purified by the same procedure. However, the purified mutant proteins used showed some impurities, representing no more than 25% of the total protein, when electrophoresed on cellulose polyacetate (Sepharose III) strips.

Spectral Measurements—Absorption spectra were measured on a Beckman DK2 spectrophotometer. Rate studies of absorbance changes were measured automatically with a Gilford model 2000 recording spectrophotometer at 37°. Uncorrected fluorescence measurements were made with an Aminco-Bowman spectrophotofluorometer at room temperature.

Assay for InGP Formation—InGP formation was detected either by the formation of indole-3-aldehyde upon treatment with periodate (8) or by the increase in absorbance at 280 µν that accompanies conversion of the aromatic ring of either PRA or CDRP to the indole ring of InGP (7) (see “Results”).

RESULTS

Due to the probable lability of PRA, it seemed that the best way to detect its presence would be by its enzymatic conversion to indoleglycerol-P, rather than by chemical tests. This conversion is catalyzed by a single enzyme from E. coli via the intermediate 1-(α-carboxyphenylamino)-1-deoxyribulose-P (7) (Fig. 1). The enzyme activity which catalyzes the Amadori rearrangement of PRA to CDRP is designated PRA isomerase (3) and that which catalyzes the conversion of CDRP to InGP

* This research was supported by Grant GB 7329 from the National Science Foundation.

1 The abbreviations used are: PRA, N-(5'-phosphoribosyl)anthranilic acid; InGP, indole-3-glycerol 5-phosphate; CDRP, 1-(α-carboxyphenylamino)-1-deoxyribulose phosphate.
is InGP synthetase (9). Mutant forms of the enzyme from *E. coli* are known which lack one while retaining the other of the two activities (10, 11). These mutationally altered enzymes allow enzymatic differentiation between PRA and CDRP. InGP will be formed from both PRA and CDRP by the normal enzyme, but will be produced only from CDRP by a mutant enzyme lacking the PRA isomerase activity. For this purpose, the three forms of the enzyme were purified by the procedure previously described (7). The enzyme lacking PRA isomerase activity was purified from mutant trp C9830 and that lacking InGP synthetase activity from mutant trp C9941 (11).

With the use of the above criterion for detecting PRA, it was found that the intermediate is rapidly formed upon mixing equal volumes of 1.0 M anthranilic acid in 95% ethanol and 1.0 M d-ribose-5-P at room temperature. Enzymatic InGP formation was determined immediately after a 1000-fold dilution of the above mixture with 0.10 M triethanolamine-HCl buffer, pH 8.6. The maximum amount of PRA was formed within 4 min after mixing the anthranilic acid and ribose-P. The amount of InGP formed indicated a yield of 30 to 40% of the theoretical maximum. At this time the mixture contained no detectable amount of CDRP. However, this Amadori rearrangement product began to appear shortly thereafter. Within an hour the concentration of CDRP was approximately half that of the PRA present.

The following experiments, the presumptive PRA was routinely prepared by allowing the reaction between anthranilic acid and ribose-P to proceed for 4 minutes at room temperature. The mixture was then diluted 100-fold with cold 0.1 M triethanolamine-HCl buffer, pH 8.6, and kept at 0° for no more than 4 hours before use. Appropriate further dilutions were made just prior to use. After dilution, no detectable CDRP was formed. The basis for the absence of any detectable isomerization of the PRA after dilution is unknown, but is most likely a result of either dilution of the ethanol or a change in pH. Control mixtures free of PRA and CDRP were prepared by individually diluting the anthranilic acid and the ribose-P solutions into the same buffer to give the equivalent mixture as above.

The ultraviolet absorption spectra of *N*-ribosylanthranilic acid (4, 5) and PRA (3) have been reported to differ significantly from that of anthranilic acid. If 30 to 40% of the anthranilic acid can be converted to PRA in the above manner, this conversion should be reflected in the ultraviolet absorption spectrum of the mixture. Fig. 2 compares the absorption spectrum of an appropriately diluted mixture containing PRA with that of an equivalent mixture in which no PRA formation has been allowed. The observed spectra are in excellent agreement with those expected (5). At room temperature or above, the spectrum of the PRA mixture changed rapidly with time and reverted to a spectrum identical with that of anthranilic acid. This behavior is consistent with the tendency of glycosylamines to break down to the amine and sugar (5, 12).

Previous observations have indicated that PRA is significantly less fluorescent than anthranilic acid (2, 3). The fluorescence spectra of the PRA mixture and the equivalent anthranilic acid mixtures were essentially identical with respect to wave length (within 2 mp for both excitation and emission), but the fluorescence yield of the PRA mixture was 30% less than that of the control mixture. This is the result expected if 30 to 40% of the anthranilic acid has been converted to a compound with greatly decreased fluorescence.

PRA should be converted to CDRP by the purified enzyme with only PRA isomerase activity. Although no significant alterations in ultraviolet absorption spectra of glycosylamines are expected upon undergoing the Amadori rearrangement (13), slight differences have been reported in the case of *N*-ribosyl-
FIG. 2. Ultraviolet absorption spectra of a PRA preparation (—) and of an equivalent mixture of anthranilic acid and ribose-P (——). The buffer was 0.10 M triethanolamine-HCl, pH 8.6. The equivalent concentration of both anthranilic acid and ribose-P was 0.40 mM. Enzymatic InGP formation indicated a 35% yield of PRA. The solutions were originally cold to stabilize the PRA, but no attempt was made to keep the temperature below ambient during the spectral measurements. Rescanning showed that during the measurement of the PRA-containing mixture, approximately 5% of the PRA had broken down.

anthranilic acids (3, 4). Any change in absorption spectra of the PRA mixture was difficult to measure due to the instability of the PRA (see below), but the spectrum was prevented from reverting to that of anthranilic acid by addition of enzyme with only PRA isomerase activity. This is as would be expected, as CDRP is considerably more stable than PRA (3). However, the stabilized spectrum was not significantly different from that of the original solution. The addition of purified enzyme with PRA isomerase activity to the PRA mixture resulted in a small, but rapid, decrease in the fluorescence yield (excitation, 310 mp; emission, 400 mp) of the mixture, which leveled off at a value only 6% less than that of the original PRA mixture. This result would indicate that at these wave lengths PRA is somewhat fluorescent and that CDRP is less fluorescent. No changes in the absorption or fluorescence spectra of the control anthranilic acid solution were detected upon addition of the same amount of enzyme.

The parent glycosylamine and its Amadori rearrangement product may be distinguished by their relative reducing activities, the rearrangement product being much more strongly reducing (13). Accordingly, CDRP has been shown to be a potent reducer of tetrazolium dyes (4, 6, 14). To this end, 2-p-iodophenyl-3-p-nitrophenyl-5-phenyltetrazolium chloride, 72 µg of phenazine methosulfate, and 0.36 mg of gelatin in a total volume of 3.0 ml. At zero time equal amounts (approximately 20 µg) of purified enzyme were added to each sample. The control for each sample was the same mixture without added enzyme. An additional control without PRA, anthranilic acid, and ribose-P showed no detectable dye reduction upon addition of the same amount of enzyme.

The over-all conversion of PRA to InGP should lead to a significant alteration in the ultraviolet absorption properties of the mixture, similar to those observed upon the conversion of CDRP to InGP (7). The results obtained after addition of purified normal enzyme with both PRA isomerase and InGP synthetase activities are illustrated in Fig. 4. The observed changes in spectra are in excellent agreement with those ex-
The rate of decrease in absorbance of freshly diluted PRA mixtures at 37° indicated that the process followed approximately first-order kinetics. The observed half-lives in several different buffers are tabulated in Table I. Semiquantitative measurements of the rate of loss of InGP precursor support the assumption that PRA loss parallels the change in absorption spectrum. The data vividly illustrate the lability of this intermediate and provide further evidence that the compound being converted to InGP is PRA, since its stability increases with increasing pH (3, 5). In contrast to the findings at 37°, the half-life of PRA at pH 8.6 and 0° is of the order of several hours.

**Table I**

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Concentration</th>
<th>pH</th>
<th>Half-life</th>
</tr>
</thead>
<tbody>
<tr>
<td>Potassium phosphate</td>
<td>0.10</td>
<td>7.06</td>
<td>2.1</td>
</tr>
<tr>
<td>Tris-HCl</td>
<td>0.10</td>
<td>7.67</td>
<td>3.5</td>
</tr>
<tr>
<td>Triethanolamine-HCl</td>
<td>0.10</td>
<td>8.02</td>
<td>6.5</td>
</tr>
<tr>
<td>Triethanolamine-HCl</td>
<td>0.10</td>
<td>8.64</td>
<td>15</td>
</tr>
</tbody>
</table>

**DISCUSSION**

The above studies have shown that an intermediate in tryptophan biosynthesis between anthranilic acid and InGP has been produced nonenzymatically. The intermediate is converted to either CDRP or InGP by highly purified preparations of the appropriate enzymes from *E. coli*. The intermediate is distinct from CDRP because it is not converted to InGP by an enzyme which is able to utilize synthetic CDRP and has shown that the compound being converted to InGP is PRA, since its stability increases with increasing pH (3, 5). In contrast to the findings at 37°, the half-life of PRA at pH 8.6 and 0° is of the order of several hours.

Further evidence that the intermediate is PRA comes from the similarity of its spectral and chemical properties to those of synthetic N-ribosylanthranilic acid (4). Moreover, a glycosylamine is the most likely product to be formed under the conditions used (12). The lability of the intermediate has not yet permitted its purification, precluding any chemical determination of its structure at this time. Based on the assumption that the intermediate is PRA, it is still unknown which of the possible structural isomers is biologically active.

Although the PRA as prepared here is unstable and is contaminated at least with unreacted anthranilic acid and ribose-P, the ease of preparation makes it very feasible to use this PRA in enzymatic studies. The development of a convenient and quantitative assay for PRA isomerase activity has been a subject of much investigation (10, 16, 17), so the availability of synthetic PRA should be of considerable use. Unfortunately, following CDRP production by reduction of tetrazolium dyes is unsuitable due to the ribose-P contamination and to the ability of most enzyme preparations to convert the CDRP to InGP. The results of this investigation suggest that the best assay for PRA isomerase activity is measurement of InGP formation from synthetic PRA, either by the increase in absorbance at 280 μm by the periodate assay (8). A suitable source of InGP synthetase activity must be added to make the conversion of PRA to CDRP the rate-limiting step.

**REFERENCES**

10. Smith, O. H., Genetics, 57, 95 (1967).
The Nonenzymatic Preparation in Solution of N-(5'-Phosphoribosyl)anthranilic Acid, an Intermediate in Tryptophan Biosynthesis

Thomas E. Creighton