Carbohydrate Metabolism in *Streptomyces hygroscopicus*

I. ENZYMATIC SYNTHESIS OF TREHALOSE PHOSPHATE FROM GUANOSINE DIPHOSPHATE D-GLUCOSE-\(^{14}C\)*

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SUMMARY

An enzyme system has been isolated from *Streptomyces hygroscopicus* that catalyzes the transfer of glucose from guanosine diphosphate D-glucose-\(^{14}C\) to glucose 6-phosphate to form trehalose phosphate. The reaction appears to be specific for GDP-glucose; relatively little activity was found in trehalose phosphate when other \(^{14}C\)-glucose nucleotides were used in place of GDP-D-glucose. Glucose-6-P, mannose-6-P, and fructose-6-P all served as acceptors because of the presence of phosphohexose isomerase, but other phosphorylated sugars were inactive. The product formed in the presence of GDP-D-glucose-\(^{14}C\) and glucose-6-P was characterized as trehalose phosphate by chemical, enzymatic, and chromatographic methods.

During the course of studies on the biosynthesis of the hygromycin antibiotics, it was found that, when guanosine diphosphate D-glucose-\(^{14}C\) was incubated with cell-free extracts of *Streptomyces hygroscopicus* and the incubation mixtures were subjected to paper electrophoresis in 0.2 M ammonium formate buffer, pH 3.6, a new radioactive peak was detected which had a mobility slower than that of GDP-D-glucose-\(^{14}C\).

In the present report, an enzyme system from *S. hygroscopicus* is described which catalyzes the synthesis of trehalose phosphate by the following reaction.

\[
\text{GDP-D-glucose} + \text{glucose-6-P} \rightarrow (\text{GDP}) + \text{trehalose-1P}
\]

The enzymatic synthesis of trehalose phosphate from uridine diphosphate D-glucose and glucose-6-P by an enzyme system from yeast (1) and later from insects (2) has been reported.

EXPERIMENTAL PROCEDURE

**Materials**—Sugar nucleotides were prepared chemically from uniformly labeled \(^{14}C\)-\(\alpha\)-D-glucose-1-P and the corresponding monophosphate of the nucleoside monophosphate (3). \(^{14}C\)-\(\alpha\)-D-Glucose-6-P was synthesized from uniformly labeled \(\alpha\)-D-glucose-\(^{14}C\) and ATP by means of crystalline yeast hexokinase (Worthington). All other compounds were obtained from commercial sources.

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mainning sugar nucleotide; then NaOH was added to a final concentration of 0.1 N. The mixtures were again heated at 100° for 10 min. This treatment destroyed free sugars, disaccharides with free carbonyl groups such as cellobiose, and phosphorylated compounds such as glucose-6-P. Trehalose and trehalose phosphate could then be assayed by the anthrone method.

FIG. 1. Paper electrophoresis of incubation mixtures. The reaction mixtures contained the following (micromoles in a final volume of 0.15 ml): 14C-labeled compound, 0.1 (10,000 cpm); glucose-6-P, 1; phosphate buffer, pH 7.5, 2; and 25 μl of the 40 to 70% ammonium sulfate fraction. After incubation for 15 min at 37°, the reaction mixtures were spotted directly on Whatman No. 3MM paper and subjected to high voltage electrophoresis in 0.2 M ammonium formate buffer, pH 3.6. Disaccharide phosphate areas of the paper were cut out and radiocounted in a liquid scintillation spectrometer. GPDG, guanosine diphosphate d-glucose.

TABLE I

Specialty of glucosyl donor for trehalose phosphate synthesis

The reaction mixtures contained the following (micromoles in a final volume of 0.15 ml): 14C-labeled compound, 0.1 (10,000 cpm); glucose-6-P, 1; phosphate buffer, pH 7.5, 2; and 25 μl of the 40 to 70% ammonium sulfate fraction. After incubation for 15 min at 37°, the reaction mixtures were spotted directly on Whatman No. 3MM paper and subjected to high voltage electrophoresis in 0.2 M ammonium formate buffer, pH 3.6. Disaccharide phosphate areas of the paper were cut out and radiocounted in a liquid scintillation spectrometer.

<table>
<thead>
<tr>
<th>14C-Labeled substrate</th>
<th>Radioactivity in trehalose-P (cpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose-6-P</td>
<td>5530</td>
</tr>
<tr>
<td>Glucose-1-P</td>
<td>80</td>
</tr>
<tr>
<td>Glucose</td>
<td>5</td>
</tr>
<tr>
<td>GDP-glucose + boiled enzyme</td>
<td>51</td>
</tr>
<tr>
<td>Glucose-6-P</td>
<td>5710</td>
</tr>
<tr>
<td>UDP-glucose</td>
<td>76</td>
</tr>
<tr>
<td>TDP-glucose</td>
<td>40</td>
</tr>
<tr>
<td>CDP-glucose</td>
<td>30</td>
</tr>
<tr>
<td>ADP-glucose</td>
<td>140</td>
</tr>
</tbody>
</table>

* Omitted when 14C-glucose-6-P was used as substrate.

TABLE II

Requirements for trehalose phosphate synthesis

The reaction mixtures contained the following (micromoles in a final volume of 0.1 ml): GDP-D-glucose-14C, 0.3 (10,000 cpm); phosphate buffer, pH 7.5, 2; sugar phosphate as indicated, 1; and 25 μl of enzyme (100 μg of protein). After incubation for 30 min at 37°, HCl was added to a final concentration of 0.2 N and the reaction mixtures were heated at 100° for 15 min. The mixtures were then spotted on paper and subjected to high voltage electrophoresis in 0.2 M ammonium formate buffer, pH 3.6. Disaccharide phosphate areas of the paper were then cut out and counted in a liquid scintillation spectrometer.

<table>
<thead>
<tr>
<th>Sugar phosphate added</th>
<th>Radioactivity in trehalose-P (cpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose-6-P</td>
<td>5741</td>
</tr>
<tr>
<td>Glucose-1-P</td>
<td>1238</td>
</tr>
<tr>
<td>None</td>
<td>583</td>
</tr>
</tbody>
</table>

RESULTS

Specificity of Reaction—As shown in Fig. 1, when GDP-D-glucose-14C was incubated with the enzyme fraction, a slower moving radioactive peak was observed on the electrophoretogram. The formation of this new radioactive material was completely dependent on the presence of active enzyme; when heat-denatured enzyme was used, no activity was found in this area (Table I). Furthermore, the enzyme system appears to be relatively specific for GDP-D-glucose as the glucosyl donor. As shown in Table I, when other 14C-glucosyl nucleotides were used in place of GDP-D-glucose-14C, only trace amounts of radioactivity were found in the disaccharide phosphate. These small amounts of radioactivity shown in Table I, with other glucosyl nucleotides, probably represent slight contamination of this area by the unhydrolyzed nucleotides or their breakdown products. It may be that the enzyme system catalyzing the synthesis of trehalose phosphate is not completely specific and is able to use other glucosyl nucleotides to a very limited extent. This latter possibility seems unlikely, however, in view of the fact that no activity could be detected with other glucosyl nucleotides when the synthesis of trehalose phosphate was determined directly by the colorimetric assay described previously. Also, as shown in Table I, when GDP-glucose-14C was replaced by either 14C-glucose-6-P, 14C-glucose-1-P, or 14C-glucose, little or no radioactivity was found in the slower moving area.

Requirements for Synthesis—The conversion of GDP-D-glucose-14C to trehalose phosphate was markedly stimulated by the addition of glucose-6-P (Table II). When glucose-6-P was omitted from reaction mixtures, only 10% as much radioactivity was found in the product. Further, glucose-1-P was only slightly effective in replacing glucose-6-P. As shown in Table III, fructose-6-P and mannose-6-P were equally effective in fulfilling the requirement for glucose-6-P. The difference in the amount of radioactivity among mannose-6-P, fructose-6-P, and glucose-6-P is within the experimental error for this assay, so that they appear to be equally effective acceptors with this enzyme fraction. However, ribose-5-P and galactose-6-P as well as mannose-1-P could not replace glucose-6-P. The fact that mannose-6-P and fructose-6-P can act as acceptors appears to be due to the presence of phosphohexose isomerase since preliminary tests have shown the presence of this enzyme in these extracts. Further, the product formed from
GDP-glucose and \(^{14}\)C-mannose-6-P was characterized as trehalose phosphate as follows. After dephosphorylation with potato acid phosphatase (Sigma) the radioactive disaccharide cochromatographed with authentic trehalose. Upon hydrolysis of this disaccharide, the radioactivity migrated at the same rate as glucose upon paper chromatography, indicating that the mannose-6-P had been isomerized to glucose-6-P.

When GDP-glucose was incubated with \(^{14}\)C-glucose-6-P and the enzyme fraction, about 50% of the radioactivity was found in the disaccharide phosphate upon paper electrophoresis. Treatment of this material with potato acid phosphatase resulted in the formation of a neutral radioactive compound which showed the same mobility as authentic trehalose upon paper chromatography in several different solvent systems (Table IV).

Characterization of Product—Characterization of the product formed from GDP-glucose-\(^{14}\)C and glucose-6-P was performed as follows: 10 \(\mu\)moles of GDP-glucose-\(^{14}\)C (345,000 cpm) and 20 \(\mu\)moles of glucose-6-P were incubated with 2 ml of enzyme in 0.05 \(\mu\)M ammonium formate buffer, pH 3.6. More than 85% of the radioactivity was recovered in the disaccharide phosphate. The radioactivity in this area was eluted from the paper and further purified either by electrophoresis in 0.2 \(\mu\)M ammonium formate buffer, pH 3.6. The phosphate group on the disaccharide phosphate was not attached to position 1.

The phosphorylated compound was treated with either potato acid phosphatase or calf mucosa alkaline phosphatase (Sigma), after which 75 to 85% of the radioactivity was no longer mobile upon electrophoresis, indicating that it had lost its charge (Fig. 2). The radioactivity remaining at the origin of the electrophoretogram shown in Fig. 2 was eluted and purified by paper chromatography in several different solvent systems. As shown in Table IV, the dephosphorylated disaccharide exhibited the same mobility as authentic trehalose in four different solvent systems.
systems. In each case, some difficulty was encountered in separating trehalose from kojibiose. However, it was possible to separate these two disaccharides by paper electrophoresis in 0.05 M sodium tetraborate, pH 9.7; the radioactive disaccharide moved only slightly from the origin, as did authentic trehalose, whereas kojibiose migrated considerably further (Table IV). Further proof that the unknown disaccharide contained a 1,1-glycosidic linkage was the fact that it did not react in the reducing sugar test (6), indicating that the carbonyl group was bound in a glycosidic linkage. In addition, the disaccharide was not destroyed by heating in 0.1 N NaOH, indicating that the unknown compound had the same stability in alkali as does authentic trehalose (1); glucose, cellobiose, and kojibiose were destroyed under these conditions.\(^1\)

Analysis of the disaccharide gave the following molar ratios: anthrone-reacting hexose, 1.00; glucose oxidase (after hydrolysis of the unknown in 3 N H\(_2\)SO\(_4\) at 100° for 30 min), 1.02; reducing sugar (on the unhydrolyzed sample), less than 0.1. Authentic trehalose also gave a ratio of anthrone-reacting hexose to glucose oxidase of about 1:1. Hydrolysis of the radioactive disaccharide liberated only D-glucose, as shown by paper chromatography (Table IV) as well as by reaction with glucose oxidase. Although GDP is presumably a product of the reaction it has not been identified as yet.

It is not yet certain which isomer of trehalose is represented by the unknown compound, that is, whether the compound formed from GDP-\(\beta\)-glucose and glucose-6-P is \(\alpha,\beta\)-trehalose phosphate, \(\beta,\beta\)-trehalose phosphate, or \(\alpha,\beta\)-trehalose phosphate. The fact that the dephosphorylated radioactive disaccharide was not hydrolyzed by \(\beta\)-glucosidase (almond emulsin, Sigma), although under the conditions used cellulobiose was completely converted to glucose, may be an indication that the disaccharide is \(\alpha,\alpha\)-trehalose. Further, \(\alpha,\alpha\)-trehalose is the only isomer of trehalose found in nature. The compound formed from GDP-\(\beta\)-glucose and \(\alpha,\beta\)-glucose-6-P is therefore believed to be \(\alpha,\alpha\)-trehalose phosphate. However, further characterization of the product is necessary before this can be definitely established.

In several cases, when incubation mixtures containing GDP-\(\beta\)-glucose-\(^{14}\)C and crude extract were subjected to electrophoresis as described (Fig. 1), some radioactivity was found at the origin. The radioactivity was shown to be in trehalose, indicating that crude extracts probably contain a phosphatase (or phosphatasas) capable of converting trehalose phosphate to trehalose. Whether this is a specific enzyme remains to be established. Studies are now in progress on the purification and characterization of the trehalose phosphate-synthesizing system.

**DISCUSSION**

The physiological significance of this reaction in *Streptomyces* is not clear at the present time, since neither trehalose nor trehalose phosphate has been isolated from these organisms. Trehalose is found in large amounts in insects (9) and fungi (10), where it is considered to be a storage compound (11); however, in *Myobacterium tuberculosis* \(\alpha\)-trehalose 6,6'-diphosphate has been characterized as a subunit of a phosphorylated polysaccharide (12).

The enzymatic synthesis of GDP-\(\beta\)-glucose has now been reported in a number of organisms, including animals (13) and plants (14). Although the synthesis of this nucleotide has not been demonstrated in *Streptomyces*, GDP-\(\beta\)-glucose itself was isolated from *Streptomyces griseus* (15). Recently, an enzyme system has been described in yeast which catalyzes the hydrolysis of GDP-\(\beta\)-glucose to GDP and glucose (16).

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**REFERENCES**