Metabolism of Trehalose in *Euglena gracilis*

I. PARTIAL PURIFICATION AND SOME PROPERTIES OF TREHALOSE PHOSPHORYLASE*

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**SUMMARY**

Trehalose phosphorylase, an enzyme found in cell-free extracts of *Euglena gracilis* var. bacillaris, was purified 75-fold by treatment with protamine sulfate, centrifugation at 200,000 × g, and chromatography in a column of hydroxyapatite. This enzyme catalyzes the reversible phosphorolytic splitting of trehalose, yielding β-glucose 1-phosphate and glucose as products. The optimum pH of the reaction was 7.0 for phosphorolysis and 6.3 for the synthesis of trehalose.

The equilibrium constant changes with pH. It was 4.2 at pH 7.0 and 17 at pH 6.3.

The enzyme is very unstable in the absence of inorganic phosphate, α- or β-glucose 1-phosphate.

Measurements in sucrose gradient gave a molecular weight of about 344,000.

This enzyme together with a phosphoglucomutase for β-glucose 1-phosphate found in the same *Euglena* extracts would constitute a new catabolic pathway for trehalose.

EXPERIMENTAL PROCEDURE

Materials—β-Glucose-1-P, dipotassium salt, was prepared as described by Reithel (12), with slight modifications. Deoxy-β-glucose was obtained by acid hydrolysis of its α-methyl glucoside, purchased from Pierce Chemical Co. Laminaribiose and laminaribiosyl 1,4-glucose were prepared as indicated by Goldemberg et al. (13).

Hydroxyapatite and calcium phosphate gel were obtained as indicated by Levin (14) and Keilin and Hartree (15), respectively.

Trehalase from rabbit kidney was purified as described by Sacktor (16). [32P]Phosphorylase a, was a generous gift from Dr. Héctor Torres (17). All other materials were commercial preparations.

Methods—Protein was determined according to the procedure of Lowry et al. (18). Total hexoses were estimated as described by Dubois et al. (19). The reducing power was measured by the Somogyi (20) and Nelson (21) methods. Glucose was estimated by the glucose oxidase-peroxidase method (22), with slight modifications. Inorganic phosphate was determined as described by Fiske and SubbaRow (23). Trehalose was analyzed by incubation with kidney trehalase and determined by the glucose liberated as mentioned above. β-Glucose 1-P was measured either by the labile phosphate (1 N HSO₄ for 5 min at 100°C) or with a specific phosphoglucomutase obtained from *Euglena* extracts coupled with glucose-6-P dehydrogenase and NADP.

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‡ Career investigator of the Consejo Nacional de Investigaciones Científicas y Técnicas (Argentina).

confirmed (10). However, working with cell-free extracts of *Euglena gracilis* we have found an enzyme which catalyzes the reversible phosphorolytic splitting of trehalose (11), according to the following reaction.

\[ \text{Trehalose} + \text{P} \rightarrow \beta\text{-glucose-1-P} + \text{glucose} \]

This enzyme was very unstable in the absence of phosphate buffer and no activity could be detected in crude or partially purified extracts when they were dialyzed against water or different buffers, with or without —SH compounds.

This paper describes the partial purification of the enzyme and some of the properties of the reaction.

The name α-D-glucopyranosyl-α-D-glucopyranose: orthophosphate glucosyltransferase is proposed for this enzyme, while trehalose phosphorylase is used as trivial name.

It is generally accepted that the biosynthesis of trehalose is carried out in two steps. In the first one, trehalose 6-phosphate synthetase (UDP-glucose:α-glucose 6-phosphate 1-glucosyltransferase, EC 2.4.1.15) catalyzes the transfer of the glucosyl moiety from UDP-glucose (1-5) or GDP-glucose (0, 7), to glucose-6-P, forming trehalose-6-P. In a second step, a specific phosphatase (trehalose 6-phosphate phosphohydrolase, EC 3.1.3.12) catalyzes the hydrolysis of the phosphate group, leaving free trehalose (1, 8).

On the other hand, trehalase (α-D-glucoside 1-glucohydrolase, EC 3.2.1.28) is the only enzyme hitherto described which is involved in the breakdown of trehalose. This enzyme catalyzes the hydrolysis of this disaccharide, yielding 2 moles of glucose. The suggestion of Frerejacque (9) about the presence in insects of a phosphorylase acting on trehalose, could not be confirmed (10). However, working with cell-free extracts of *Euglena gracilis* we have found an enzyme which catalyzes the reversible phosphorolytic splitting of trehalose (11), according to the following reaction.

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¹ E. Belocopitow, and L. R. Maréchal, to be published.
Enzyme Assay—The enzymatic activity was usually measured in the direction of trehalose phosphorolysis (Reaction 1). Unless otherwise specified, the incubation mixtures contained 50 mM imidazole-HCl buffer at pH 7.0; 50 mM phosphate buffer at pH 7.0; 50 mM trehalose and enzyme, in a total volume of 40 nl. Incubations were carried out at 37° for 30 min. The reaction was stopped by adding 0.5 ml of Somogy's reagent, and the amount of the reducing power liberated was measured as indicated above.

When the reaction was measured in the direction of trehalose synthesis, the incubation mixtures contained 50 mM imidazole-HCl buffer at pH 7.0; 25 mM \( \beta \)-glucose-1-P; 125 mM glucose and enzyme, in a total volume of 40 nl. The reaction mixtures were incubated at 37° for 30 min and the released Pi was measured as indicated above. When the activity was tested in crude extracts, the presence of Pi was previously deproteinized with 5% cold trichloroacetic acid.

RESULTS

Enzyme Preparation—Cells of \( E. \) gracilis var. bacillaris, originally obtained thanks to a generous gift from Dr. D. E. Buettow and Dr. W. F. Danforth, were grown under natural light at room temperature, in a medium of the following composition: 0.5 g of peptone, 0.2 g of yeast extract, 1 ml of ethanol, 3 \( \mu \)g of vitamin B\( _{12} \), and 100 ml of water. A 5-ml inoculum prepared from an agar slant was grown for a week and added to 200 ml of culture in a Roux bottle. After 6 days incubation, it was used as inoculum for 15 liters of culture medium, with forced aeration. After another 6 days, the cells were collected in an Alfa-Laval centrifuge. The following steps were carried out at 0-4°. The cells were washed repeatedly with water by centrifugation at 3,000 \( \times \) \( g \), and either frozen at \(-70^\circ\) or immediately processed. To prepare the crude extracts, the packed cells were resuspended in 1 to 1.5 volumes of a solution containing 2 mM EDTA, 4 mM phosphate buffer, 25% glycerol, at pH 7.0, and disrupted in a French press at 12,000 p.s.i. ("crude extract").

The latter extract was centrifuged at 5,000 \( \times \) \( g \) for 15 min, and the residue was washed with the same solution used for disrupting the cells. This process was repeated twice and the final pellet was discarded. The supernatants were pooled, and a protamine sulfate solution was added dropwise (about 150 \( \mu \)g per mg of protein). This suspension was maintained on ice for 15 min, with occasional stirring, then centrifuged at 25,000 \( \times \) \( g \), and the supernatant was collected ("Protamine Sp").

In order to obtain a maximal trehalose phosphorylase activity in the supernatant, it was necessary to run a small scale assay because an excess of protamine solution precipitates this enzyme.

The Protamine Sp was centrifuged at 200,000 \( \times \) \( g \) for 3 hours in a Spinco preparative ultracentrifuge. The clear supernatant was discarded, and the resulting precipitate was dissolved in about one-tenth of the original volume with a solution containing 3 mM phosphate buffer-2 mM EDTA-25% glycerol at pH 7.0 ("200,000 pellet"). A fraction of the 200,000 pellet (about 2.5 ml) was used in phosphate buffer at pH 7.0, and applied to a column, 1.7 \( \times \) 5 cm, of a mixture of hydroxylapatite and cellulose powder (2:1 w/w, dry weight) previously equilibrated with 0.1 M phosphate buffer, pH 7.0-2 mM EDTA-25% glycerol. This column was washed with 10 ml of the latter solution, and the adsorbed protein was eluted with 10 ml of 0.15 M and 20 ml of 0.30 M sodium phosphate, pH 7.0, in 25% glycerol. The flow rate was adjusted to about 12 ml per hour with pressure from a double bulb rubber pump, and fractions of 1.0 ml were collected (Fig. 1).

The fractions with higher specific activity were pooled, and precipitated by the addition of 2.2 volumes of a saturated ammonium sulfate solution, at 0°, containing 4 mM phosphate buffer at pH 7.0-4 mM EDTA. After standing on ice for 20 min, the suspension was centrifuged at 20,000 \( \times \) \( g \) for 10 min. The resulting precipitate was dissolved in a minimal amount of a solution containing 2 mM potassium phosphate-25% glycerol. It was then dialyzed overnight against the same solution ("HAP step").

A summary of the purification is presented in Table I.

The enzyme corresponding to HAP step was free from laminariobise phosphorylase, \( \beta \)-1,3-oligoglucan phosphorylase, and phosphoglucomutases for \( \alpha \)-and \( \beta \)-glucose-1-P.

Reaction Products—\( \beta \)-Glucose-1-P was prepared and isolated as previously indicated (11), and identified as follows.

The enzyme did not reduce the Somogy's reagent; it was readily hydrolyzed at 100° for 10 min with 0.1 N HCl, giving an equimolecular amount of P\( _1 \) and glucose.

The compound ran with the same mobility as authentic \( \beta \)-and \( \alpha \)-glucose-1-P in paper chromatography with isobutyric acid-1 N \( \text{NH}_4 \)-0.1 M EDTA (50:30:1). Moreover, the infrared spectrum of the enzymatic hexose phosphate in the fingerprint zone was similar to the one obtained with the standard \( \beta \)-glucose-1-P, and different from the \( \alpha \) anomer (24).

Neither the synthetic \( \beta \)-glucose-1-P nor the product from the

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**Table I**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Volume</th>
<th>Enzyme activity glc/mg</th>
<th>Protein content</th>
<th>Specific activity</th>
<th>Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. Crude extract</td>
<td>185</td>
<td>198</td>
<td>69</td>
<td>2.55</td>
<td>1</td>
</tr>
<tr>
<td>II. 5,000 Sp</td>
<td>250</td>
<td>104</td>
<td>11.1</td>
<td>9.4</td>
<td>3.03</td>
</tr>
<tr>
<td>III. Protamine Sp</td>
<td>230</td>
<td>91</td>
<td>11</td>
<td>8.4</td>
<td>2.95</td>
</tr>
<tr>
<td>IV. 200,000 pellet</td>
<td>20</td>
<td>715</td>
<td>44.5</td>
<td>16</td>
<td>5.6</td>
</tr>
<tr>
<td>V. &quot;HAP step&quot;</td>
<td>2.4</td>
<td>1150</td>
<td>19.5</td>
<td>214</td>
<td>75</td>
</tr>
</tbody>
</table>
enzymatic action were transformed into stable phosphate with muscle phosphoglucokinase, but they could be converted into glucose-6-P by using a protein fraction from Euglena extracts.\(^1\) Glucose-6-P was identified by the glucose-6-P dehydrogenase and NADP system, and by paper chromatography in ethanol-methyl ethyl ketone-0.5 M morpholinium tetahydroxide, pH 8.6, in 0.01 M EDTA (70:20:30), which differentiates glucose-1-P from glucose-6-P (25).

To identify the enzymatically obtained trehalose, it was previously prepared and isolated as follows.

A large preparative reaction mixture containing 100 \(\mu\) moles of imidazole HCl buffer at pH 7.0; 50 mg of glucose; 100 mg of 2-glucose-1-P, and enzyme (200,000 pellet), was incubated under toluene for 2 hours at 37\(^\circ\)C in a total volume of 2.8 ml. After deproteinization by heating for 5 min at 100\(^\circ\), the precipitate formed was centrifuged off and the supernatant was passed through a column of charcoal-celite (0.7 \(\times\) 5 cm). It was then washed with water, and then with 1% ethanol until no reducing power was detected with the Somogyi’s reagent. The sugar was then eluted with 10% ethanol and the solution was evaporated to racnio. The residue was dissolved in 1 ml of water, and the yellowish solution was passed through a column of Amberlite MB-3. The percolate was again evaporated to dryness as above, and redissolved in water. A sample of authentic \(\alpha\)-trehalose, used as control, was similarly treated. The amount of the enzymatic disaccharide recovered was 14 mg, as calculated by the phenolsulfuric acid method.

The disaccharide thus isolated did not reduce the alkaline copper reagent, but was readily hydrolyzed by a specific kidney trehalase (18) giving only glucose as a product, as determined by paper chromatography and the glucose oxidase-peroxidase method. In paper and thin layer chromatography, and in paper ionophoresis, it had the same mobility as authentic trehalose, and they were reduced by alkaline AgNO\(_3\) (26) at the same slowness. When the enzymatically obtained disaccharide was hydrolyzed with 1 \(\times\) SO\(_4\)H at 100\(^\circ\), the rate of glucose formation was equal to that obtained with standard trehalose (11).

The rotatory power was \([\alpha]_D^20 178.1^\circ\). It was the same as \(\alpha\)-trehalose (27).

**Enzyme Properties: Stability**—The trehalose phosphorylase activity was lost when extracts were desalted by dialysis or passage through Sephadex G-25 in the presence of water, with or without -SH compounds. When dialysis was carried out against 5 \(\times\) 10\(^{-3}\) M NaCl, glucose-6-P, EDTA, or imidazole-HCl buffer, pH 7.0, the enzymatic activity was maintained for only a few hours. Glycerol (25\%) had better stabilizing effects than the above indicated compounds.

Stabilization was obtained when the enzyme was kept in the presence of 2 \(\times\) 10\(^{-4}\) M glucose-6-P, EDTA, or imidazole-HCl buffer, pH 7.0. The enzymatic activity was maintained under these conditions, the enzyme retained full activity during 5 months at -14\(^\circ\) with several freezings and thaws.

Once the enzyme was inactivated, attempts to reactivate it were unsuccessful with several concentrations of potassium phosphate buffer, 2- or \(\alpha\)-glucose-1-P, ATP-Mg\(^{++}\), and -SH compounds.

**Effect of Enzyme and Substrate Concentrations on Reaction Rates**—Fig. 2 shows the relationship between enzyme concentration and the amount of product in both directions of reaction 1. The data show that the assays were linear up to 2 to 5\% of transformed substrates.

Double reciprocal plots (not shown) on velocity versus substrate concentration for \(P_i\), \(\beta\)-glucose-1-P, glucose, and trehalose were linear, indicating kinetics of the Michaelis-Menten type. The apparent \(K_m\) of the four substrates calculated from these plots is shown in Table II.

**TABLE II**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>(K_m) ((\mu)M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trehalose</td>
<td>33</td>
</tr>
<tr>
<td>(P_i)</td>
<td>9.4</td>
</tr>
<tr>
<td>(\beta)-Glucose-1-P</td>
<td>6</td>
</tr>
<tr>
<td>Glucose</td>
<td>32</td>
</tr>
</tbody>
</table>

Reaction 1. The data show that the assays were linear up to 2 to 5\% of transformed substrates.

The conditions of incubation were as indicated under “Methods” with an enzyme from HAP step.
The rates for forward and reverse reactions have different pH optima. For 3 hours (see "Enzyme Preparation") could indicate a rather fact that about 60% of the trehalose phosphorylase activity was recovered in the precipitate after a 200,000 x g centrifugation. Thus, at 111-16.3, the $K_{\text{eq}}$ is 17. In this manner the higher equilibrium ratio with lower $\phi\text{l}$, is similar to that obtained with polysaccharide phosphorylases (29). The variation of the $K_{\text{eq}}$ with the $\phi\text{l}$ has been predicted for those reactions where organic and inorganic phosphates are involved (30, 31). In fact, trehalose phosphorylase has also different equilibrium constants at different pH values. Thus, at pH 6.3, the $K_{\text{eq}}$ is 17. In this manner the higher equilibrium ratio with lower pH is similar to that obtained with polysaccharide phosphorylases.

From the above results, it is reasonable to suppose that the rates for forward and reverse reactions have different pH optima.

**Sucrose Gradient Studies on Trehalose Phosphorylase**—The fact that about 60% of the trehalose phosphorylase activity was recovered in the precipitate after a 200,000 x g centrifugation for 3 hours (see "Enzyme Preparation") could indicate a rather high molecular weight for the enzyme. Some experiments by gel filtration seem to confirm this assumption because the enzyme was excluded from Sephadex G-200. A sucrose gradient centrifugation for estimating the molecular weight of the protein was performed according to the procedure of Martin and Ames (32).

Fig. 4A depicts the results obtained with an enzyme purified by hydroxylapatite. The molecular weight estimated, with phosphorylase a (33) and catalase (34) as internal or external protein standards, was about 344,000. This figure also shows that the enzyme formed a rather sharply defined band in the gradients, indicating little or no heterogeneity in the sample. However about 40% of the enzyme remained in the supernatant fluid after 200,000 x g centrifugation (see "Enzyme Preparation"). This could be due to heterogeneous sizes of enzymes. To investigate this possibility, a similar experiment was performed with the supernatant of protamine sulfate treatment, i.e. without 200,000 x g centrifugation (Fig. 4B). The resulting enzyme profile, as well as the estimated molecular weight, were analogous to the more purified enzyme (Fig. 4A), suggesting that, if different isoenzymes were present, there is no gross difference in molecular weight among them.

Another probability to be taken into consideration is the binding of trehalose phosphorylase to heavy cellular components. Preliminary results seem to indicate that, if such is the case, those components are not nucleic acids, for incubations of enzyme extracts with RNase and DNase did not change the pattern of sedimentation.
FIG. 4. Sucrose density gradient. Centrifugation of extracts containing trehalose phosphorlases. A, extracts from HAP step obtained as indicated in text (0.07 ml, 0.4 mg of protein), catalase (0.02 ml, 30 mg per ml), and $[^3P]$phosphorylase a (0.02 ml, 100,000 cpm) in 50 mM phosphate buffer-2 mM EDTA at pH 7.0, in a total volume of 0.15 ml, were layered on 4.6 ml of a 5 to 30% sucrose gradient containing 50 mM phosphate buffer-2 mM EDTA at pH 7.0. Centrifugation was performed in a Spinco model L-2 preparative ultracentrifuge with SW 50 rotor at 50,000 rpm for 3 hours at 4°C. Fractions of approximately 0.12 ml were collected from the bottom of the tubes and samples were taken for assays of the different enzymatic activities as follows: trehalose phosphorylase (••••) as indicated under “Methods” in the phosphorolytic direction; catalase ($\Delta_{425}$nm, min$^{-1}$), by incubating it in the presence of excess H$_2$O$_2$, and measuring the remaining H$_2$O$_2$ with potassium iodide solution, according to the method of Terenzi et al. (35); $[^3P]$phosphorylase a (■■■■), by measuring the radioactivity present in the different fractions with a Packard Tri-Carb liquid scintillation spectrometer, with Bray’s solution. B, extracts from Protamine Sp, obtained as indicated in text (0.14 ml, 3.2 mg of protein), catalase (4 ml, 30 mg per ml), and alcohol dehydrogenase (6 ml, 10 mg per ml) in 50 mM phosphate buffer, 2 mM EDTA at pH 7.0, in a total volume of 0.15 ml, was run as described in Fig. 4A. Alcohol dehydrogenase (□□□□) assayed as described by Raeker (36).

FIG. 5. Possible steps in trehalose metabolism in E. gracilis. 1, trehalose phosphorylase. 2, phosphoglucomutase for $\beta$-glucose-1-P. NDP, nucleoside diphosphate; NTP, nucleoside triphosphate. Broader arrows show the hypothetical role of these enzymes in the catalysis of trehalose in E. gracilis.

It is now believed that the physiological processes involving the synthesis of oligo- and polysaccharides take place by means of the action of transferases, with nucleoside diphosphate sugars as glycosyl donors, while phosphorylases and hydrolyses act as degradative enzymes. The breakdown of oligo- and polysaccharides by hydrolytic enzymes involves an expenditure of
energy higher than that observed with phosphorylases. The only enzyme hitherto known, which catalyzes the degradation of trehalose, is trehalase. Trehalose phosphorylase would permit, at least in Euglena, to conceive a more conservative pathway, since the energy of the glycosidic bond is transferred to the hexose phosphate and therefore not lost. Besides, we could never detect the presence of trehalase in the extracts obtained from E. gracilis. It should here be noticed that Manners and Taylor could not obtain any hydrolysis of αα-trehalose with cell-free extracts of another protist, Astasia acellata (39).

It is interesting to consider the fate of β glucose 1-P produced in the phosphorylase of trehalose. In the Euglena extracts we have found an activity which transforms β glucose 1-P into glucose-6-P! The action of this enzyme would enable the connection of the β-glucose-1-P formed, with the known pathways of glucose metabolism. Trehalose phosphorylase and phosphoglucomutase for β-glucose-1-P would be catalytic constituents for a new catabolic pathway for trehalose. A similar case was studied for maltose in Neisseria meningitidis (40). Fig. 5 shows the different steps of metabolism for trehalose, which we assume occur in E. gracilis. The left side would indicate the probable steps in its synthesis, as described in other organisms.

Acknowledgments—We are grateful to Dr. D. E. Buetow and Dr. W. F. Danforth, for their generous gift of a photosynthetic strain of E. gracilis var. bacillaris; to Dr. E. A. Ruvels, for carrying out the determination of the infrared spectra for /I-glucose-1-P, and to Dr. A. Cerezo for the polarimetric measure of trehalose. We are particularly indebted to Dr. L. F. Leloir for his invaluable guidance and support, and to all of the other members of the Instituto de Investigaciones Bioquímicas “Fundación Campomar” for their helpful discussions on this work.

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