Phosphoenolpyruvate-dependent Fructose Phosphorylation in Photosynthetic Bacteria

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SUMMARY

The non-sulfur purple photosynthetic bacteria, Rhodospirillum rubrum and Rhodopseudomonas spheroides, utilize fructose via a pathway initiated by an enzyme system that catalyzes the transfer of phosphate from phosphoenolpyruvate to the 1-hydroxyl of fructose. Two protein fractions, both required for phosphorylating activity, were separated. One is tightly associated with the membrane fraction, while the other, found in this fraction in the crude extract, was solubilized by extraction with water, and exhibits a molecular weight near 200,000. The enzyme system differs from the P-enolpyruvate-dependent phosphotransferase systems isolated from other bacteria.

A phosphoenolpyruvate-dependent phosphotransferase system required for the translocation of a variety of sugars across the plasma membrane (1, 2) is found in obligate and facultative anaerobic bacteria (3). Detailed analysis of the PTS from Escherichia coli, Salmonella typhimurium, and Staphylococcus aureus (1, 4, 5) has shown that the phosphorylation of a given sugar requires a sugar-specific membrane component, the Enzyme II complex, and two soluble proteins that lack sugar specificity. Enzyme I and HPr. The reactions catalyzed by the system are as follows:

Phosphoenolpyruvate + HPr → Enzyme I

Enzyme I Phospho-HPr + pyruvate → Phosphoenolpyruvate + HPr + pyruvate

Enzyme II sugar + complex → sugar-P + HPr + HPr

The abbreviations used are: PTS, phosphoenolpyruvate-phosphotransferase system; PEP, phosphoenolpyruvate; HPr, histidine-containing phosphotransfer protein; fructokinase, a sugar-specific membrane component of the Rhodopseudomonas spheroides PTS; HPr, the particulate component of the R. rubrum PTS.

Phosphoenolpyruvate-dependent sugar phosphorylation has not been reported in a photosynthetic organism. In view of a recent report that sugar uptake in Chlorella can be driven by light (6), a study was initiated to determine whether a PTS was present in photosynthetic bacteria, and whether it was associated with the photosynthetic apparatus. The present communication presents a preliminary characterization of a phosphoenolpyruvate-sugar phosphotransferase found in Rhodospirillum rubrum and Rhodopseudomonas spheroides. The system is highly specific for only one of several sugars tested. Further, only two protein fractions are required for sugar phosphorylation. In contrast to the PTS isolated from other bacteria, both activities are associated with the particulate fraction under physiological conditions and no evidence was obtained for a low molecular weight phosphate carrier protein, such as HPr (1, 4).

These properties suggest that the reactions catalyzed by this new system may differ in mechanism from those previously studied.

R. rubrum, obtained from Dr. E. N. Moundrianakis (of this department) was grown in the dark in Medium 63 without iron (7) supplemented with 1% bacto-peptone, 0.5% yeast extract, and 0.2% D-fructose. Fructose increased the growth rate and yield of cells, and enhanced the activity of the phosphorylating system from 2- to 4-fold. R. spheroides, obtained from Dr. Jane Gibson, Department of Microbiology, Cornell University, was grown in a medium containing only 0.4% yeast extract, 0.6% casamino acids, and 0.2% fructose. Cells were harvested in the late exponential phase of growth, washed with Medium 63, and either resuspended for transport studies, or ruptured by passage through a French Pressure Cell. Transport and enzyme analyses were conducted as previously described (8).

The strain of R. rubrum employed for the present studies utilized fructose for growth but not other sugars tested (Table I). Uptake studies with radioactive sugars revealed that fructose was not the only sugar taken up by the cells (Table I). However, only fructose continued to be taken up linearly after the 1st min of the experiment. The inability of these cells to utilize glucose remains unexplained, since the extracts contained an ATP-dependent glucokinase.

The evidence, described below, leading to the conclusion that R. rubrum contains a PEP-dependent phosphotransferase system that phosphorylates fructose, can be summarized as follows: (a) PEP was 10 times more effective than ATP as a phosphoryl donor for fructose, whereas ATP was a much more effective phosphoryl donor for glucose (Table I); (b) the PEP-dependent and ATP-dependent activities for fructose phosphorylation were separable by simple fractionation procedures; (c) an isotope experiment showed that free ATP was not an intermediate in the transfer of the phosphoryl group from PEP to fructose; (d) the fructose-P formed from PEP was fructose-1-P and not fructose-6-P.

R. rubrum cells, suspended in 2 mM potassium phosphate buffer, pH 7, containing 0.14 M KCl and 1 mM dithiothreitol, were ruptured by passage through a French Pressure Cell; the cell-free extract was centrifuged for 2 hours at 200,000 × g. More than 90% of the PEP-dependent fructose phosphorylating activity was found in the particulate fraction; addition of the soluble to the particulate fraction stimulated phosphorylation only slightly. Over 80% of the ATP-dependent fructokinase activity was found in the supernatant fraction. The particulate fraction was sequentially extracted by resuspension and centrifugation, first in

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Dissociation of the R. rubrum PEP-dependent fructose-specific PTS into soluble and particulate components

An extract (25 mg of protein per ml) was prepared from R. rubrum cells in 2 mm potassium phosphate buffer, pH 7, containing 1 mm dithiothreitol and 0.14 M KCl as described in the text. Operations were conducted between 0° and 8°, and centrifugations were performed at 200,000 X g for 2 hours. An aliquot (5 ml) of the suspension was centrifuged (first KCl extraction), giving a supernatant and particulate fraction, and the latter was sequentially extracted with one 5-ml portion of the phosphate-KCl-dithiothreitol solution and two 5-ml aliquots of a multichannel buffer in water. In each case, the soluble and particulate fractions were separated by centrifugation, and both fractions were assayed for R-S and R-P activities. Incubation mixtures contained the following components: [14C]fructose (2.5 mCi), PEP (10 mM), potassium phosphate buffer, pH 7.4 (50 mM), MgCl2 (5 mM), potassium fluoride (10 mM), dithiothreitol (1 mm), and enzyme preparations. Assays for R-S activity were conducted with an excess of a three times water-washed membrane preparation (1.5 mg per ml). R-P activity was assayed in the presence of excess partially purified R-S (0.5 mg per ml). Incubations were at 37° for 30 min, and fructose phosphorylation was measured as described (4). Values are expressed as percentage of the protein, total recovery.

| Sugar          | Utilization for growth | Uptake in 1 min | Phosphorylation in 15 min | Phosphorylation in 30 min |
|----------------|------------------------|-----------------|--------------------------|--------------------------|
| Glucose        | −                      | 3               | 8                        | 800                      |
| Methyl α-glucoside | −                    | <1              | <1                       | <5                       |
| Fructose       | +                      | 4.5             | 70                       | 70                       |
| Mannose        | −                      | 2               | <5                       | <5                       |
| Manitol        | −                      | 0               | 0                        | <5                       |

Values are expressed as percentages of the protein, R-S, or R-P in the crude extract. The specific activity of the crude extract for fructose phosphorylation is given in Table I.

| Step                        | Protein content of | R-S activity in | R-P activity in |
|-----------------------------|-------------------|-----------------|-----------------|
|                             | Pellet            | Supernatant     | Pellet          | Supernatant |
| First KCl extraction        | 76                | 22              | 80              | 12          |
| Second KCl extraction       | 74                | 4               | 79              | 5           |
| First water extraction      | 57                | 22              | 22              | 5           |
| Second water extraction     | 52                | 4               | 12              | 89          |
| Total recovery              | 52                | 52              | 2               | 89          |

The phosphate buffer KCl and dithiothreitol mixture described above, and then twice in 1 mm dithiothreitol in water. The results of the extraction procedure are summarized in Table II. Whereas the salt wash had almost no effect on the activity of the particulate fraction, the two water washes removed a component from the particles which was required in combination with the pellet to transfer phosphate from PEP to fructose. The dissociation of the soluble component from the particulate fraction was reversible, but depended on the ionic strength; reassociation was achieved by incubating the two fractions for 30 min in the presence of 0.2 M KCl at 30°. The soluble fraction was designated R-S, while the particulate fraction was designated R-P.

Kinetic studies showed that the pH optimum with PEP as the phosphate donor was 7.6, and that Mg2+ stimulated the reaction. The rate of phosphorylation was constant with time of incubation to 30 min. With the crude extract the rate was not proportional to protein concentration, but the curve was sigmoidal. The latter results are explained by the fact that two protein fractions, R-S and R-P, are required for phosphorylation. When the rate was studied as a function of the quantity of the R-S fraction in the presence of excess R-P, and vice versa, activity plots were hyperbolic. Thus, either fraction could be made the rate-limiting component of the phosphorylating reaction.

The solubilized factor was partially purified by the extraction steps described in Table II, by ammonium sulfate precipitation, and by gel filtration through Bio-Gel P-200. The factor, R-S, penetrated the gel and was eluted from the column as a single peak. The molecular weight of R-S was estimated by filtration through a Bio-Gel P-200 column calibrated with enzymes of known molecular weight. The activity eluted immediately after muscle catalase (225,000 daltons) to give an approximate molecular weight of 200,000. No evidence was obtained for more than one soluble protein component required for phosphorylation.

The R-S and R-P fractions were sensitive to N-ethylmaleimide and to heat. A 20-min exposure to 10 mM N-ethylmaleimide at pH 8.2 and 37° completely abolished both activities, as did heating at 90° for 5 min. In addition, R-P (but not R-S) was irreversibly destroyed by a 10-min exposure to high intensity light (200 watt at a distance of 6 inches at 24°).

Partially purified R-S and a three time water washed R-P preparation were used to study the transfer of phosphate from PEP to fructose, with the use of nonradioactive PEP plus [32P]fructose, or [32P]PEP plus nonradioactive fructose. In both cases, the transfer of phosphate to sugar required the two substrates and both the R-S and R-P fractions. Treatment of the radioactive products with alkaline phosphatase gave inorganic phosphate and fructose, identified by paper chromatography and electrophoresis, as the only radioactive compounds. With either radioactive substrate, under the conditions described in Table II, the transfer reaction was not affected by addition of 5 mM ATP to the assay tube; 1 mM ADP caused slight inhibition. These results serve to characterize the reaction as a phosphoryl transfer reaction, and show that free ATP is not an intermediate in the reaction. Other potential phosphate donors, including GTP, UTP, CTP, acetyl phosphate, and the 6- and 1-phosphate esters of glucose and fructose, did not replace PEP.

PEP-dependent fructose phosphotransferases yield fructose-1-P (9-11), while known ATP-dependent hexokinases from bacteria, including a specific fructo(manno) kinase in E. coli, yield fructose-6-P (12, 13). In the following experiments the fructose-1-P synthesized by R. rubrum was isolated and compared with authentic fructose-1-P and fructose-6-P, all samples being labeled with 3H at C-1. The fructose phosphates were incubated with an excess of crystalline phosphoglucone isomerase under standard conditions (14). The isomerase catalyzes the interconversion of fructose-6-P and glucose-6-P, and the hydrogen atoms at C-1 exchange with water. More than 95% of the radioactive activity was released from [1-3H]fructose-6-P but less than 5% was released from either authentic [1-3H]fructose-1-P or the [1-3H]fructose-1-P formed by the R. rubrum phosphotransferase system, suggesting that the latter was fructose-1-P. This conclusion was also supported by periodate oxidation studies. These were conducted with the three labeled samples of fructose-1-P (4). As expected, essentially all of the label from standard [1-3H]fructose-6-P became volatile, while the radioactivity from
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