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Photosynthesis in Rhodospirillum rubrum III. Metabolic Control of Reductive Pentose Phosphate and Tricarboxylic Acid Cycle Enzymes[1]

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Summary. Enzymes of the reductive pentose phosphate cycle including ribulose-diphosphate carboxylase, ribulose-5-phosphate kinase, ribose-5-phosphate isomerase, aldolase, glyceraldehyde-3-phosphate dehydrogenase and alkaline fructose-1,6-diphosphatase were shown to be present in autotrophically grown *Rhodospirillum rubrum.* Enzyme levels were measured in this organism grown photo- and dark heterotrophically as well. Several, but not all, of these enzymes appeared to be under metabolic control, mediated by exogenous carbon and nitrogen compounds. Light had no effect on the presence or levels of any of these enzymes in this photosynthetic bacterium.

The enzymes of the tricarboxylic acid cycle and enolase were shown to be present in *R. rubrum* cultured aerobically, autotrophically, or photoheterotrophically, both in cultures evolving hydrogen and under conditions where hydrogen evolution is not observed. Light had no clearly demonstrable effect on the presence or levels of any of these enzymes.

In the previous 2 papers (1, 2) we have shown that the photometabolism of CO_2 and various other carbon compounds in the facultative photosynthetic bacterium *Rhodospirillum rubrum* is under strict metabolic control. The reductive pentose phosphate cycle operates at significant levels only under strict autotrophic conditions of growth. Yet all previous work on the photosynthetic carbon metabolism of *R. rubrum* has been done with cells grown on either acetate or malate.

Although work proposing both an anaerobic tricarboxylic acid cycle (10) and a reductive pentose phosphate cycle (1) in *R. rubrum* has been carried out, the levels of the various enzymes involved in these reactions in this organism have not been measured under any conditions of growth. It is not at all clear whether all of the enzymes of the reductive pentose phosphate cycle function in photosynthetic bacteria or if the properties of the enzymes are similar to those in plants and algae. Previously very high levels of the specific activity of ribulose 1,5-diP carboxylase in the obligate anaerobic photosynthetic bacteria *Chromatium* have been reported (9). There appears to be no structural association of this enzyme with the so-called fraction I as occurs in higher plants and algae (L. Anderson, unpublished observations).

With the above considerations in mind, a survey of some of the key enzymes of both the reductive pentose phosphate and the tricarboxylic acid cycles and their levels of activity as affected by light and various metabolic variables was undertaken.

Materials and Methods

Culture of Organisms. R. rubrum, strain S-1, was grown on the same basic media which have been described previously (1, 2). Autotrophically, cells were grown in Fernbach flasks under an atmosphere of 1 % CO_2 in hydrogen, connected, through a sterile filter, to a 1 % CO_2 buffer prepared as described by Pardee (21). Nitrogen source was 2.5 mM $(NH_4)_2SO_4$. A 5 % inoculum was used to initiate growth. The temperature, not strictly controlled, was 25°. The light source consisted of a single General Electric reflector flood 75W, 120V lamp.

When used as a carbon source L-malate was 25 mM. The nitrogen source was 2.5 mM $(NH_4)_2SO_4$ or 5 mM L-glutamate, as indicated. A 1 % inoculum was used to initiate growth. Acetate, when used as a carbon source, was 50 mM, with the nitrogen source being 2.5 mM $(NH_4)_2SO_4$. A 5 % inoculum was used to initiate growth. In an effort to maintain a constant pH cells cultured

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photoheterotrophically were bubbled with 1 % CO_2 in He; the final pH of the media was around 8.0. Under these conditions the apparent growth curve of cells cultured on L-malate, ammonium sulfate was altered. Growth as measured by turbidity was maintained at a constant rate after depletion of the nitrogen source; this was due to the deposition of polysaccharide and polyhydroxybutyrate and not to an increase in cell numbers. No difficulty was encountered in growing cells on acetate, ammonium sulfate media bubbled continuously with 1 % CO_2, He. There was considerable deposition of storage products in acetate cells. The apparent growth curve of cells cultured on L-malate, glutamate media was not altered.

Dark heterotrophic cultures were grown on 50 or 25 mM L-malate, 2.5 mM $(NH_4)_2SO_4$ under 1 % CO_2 in air at 30°. A 10 % inoculum of dark, aerobically cultured cells was used to initiate growth.

Preparation of Extracts. Cells from the logarithmic phase of growth were harvested by centrifugation, washed 2 times with 50 mM potassium phosphate buffer, pH 7, suspended in an appropriate volume of 100 mM tris or 50 mM potassium phosphate buffer, pH 7.6, as indicated, and released from 15,000 psi through a French pressure cell. The resultant extract was centrifuged for 10 minutes at 10,000 × *g* and used for assays of tricarboxylic acid cycle enzymes. For other assays the crude extract was centrifuged for an additional 30 minutes at 100,000 × *g*. All of the specific activities reported here are based on the protein content of the crude extract.

Assay Methods. A Cary 14 recording spectrophotometer was used for direct spectrophotometric assays. Other optical density measurements were made with a Zeiss spectrophotometer.

Ribulose 1,5-diP carboxylase was measured according to the method of Racker (25). The reaction mixture contained, in 1 ml, 0.15 μmole NADH, 5 μmoles GSH, 0.5 μmole ribulose 1,5-diP, 12 μmoles ATP, 10 μmoles $MgCl_2$, 75 μmoles $NaHCO_3$, 50 μmoles tris, pH 7.8, excess glyceraldehyde 3-P dehydrogenase and 3-phosphoglyceric acid kinase, and 100,000 × *g* supernatant fraction.

Phosphoribulokinase activity was measured spectrophotometrically (9). The cuvette contained, in 1 ml, 30 μmoles tris, pH 7.5, 10 μmoles $MgCl_2$, 5 μmoles GSH, 0.33 μmole ATP, 0.33 μmole phosphoenolpyruvate, 0.2 μmole NADH, 0.4 μmole ribulose 5-P, and 2.8 μmole ribose 5-P (prepared by heating ribose 5-P at 37° for 15 minutes in the presence of partially purified bacterial isomerase), excess lactic dehydrogenase and pyruvic kinase, and 100,000 × *g* supernatant.

Phosphoriboisomerase was assayed by the method of Axelrod and Jang (5).

Glyceraldehyde 3-P dehydrogenase was measured by the method of Wu and Racker (30). The reaction mixture was 5 mM with respect to 3-phosphoglycerate. The 100,000 × *g* supernatant fluid used in the assay was prepared in phosphate buffer.

Aldolase was measured using the spectrophotometric method of Wu and Racker (30) modified to permit the addition of Fe^{2+} according to Dr. J. M. Willard (unpublished). The reaction mixture contained 50 μmoles tris, pH 7.4, 0.105 μmole NADH, 2 μmoles fructose 1,6-diP, 1 μmole $Fe(NH_4)_2(SO_4)_2$, 5 μmoles cysteine, excess α-glycerophosphate dehydrogenase and glyceraldehyde 3-P isomerase, and 100,000 × *g* supernatant fraction prepared in phosphate buffer.

Fructose 1,6-diPase was measured as described by Smillie (25). Neutral fructose 1,6-diPase was measured in a reaction mixture containing, per ml, 5 μmoles $MgCl_2$, 100 μmoles tris, pH 7.3, 5 μmoles fructose 1,6-diP, and 100,000 × *g* supernatant prepared in tris buffer. Assay mixture for the alkaline phosphatase was identical except the pH of the tris buffer was 9.0. The mixture was incubated at 25° and samples removed at 3 minute intervals (for 12 mins). Inorganic phosphate was measured by the Taussky and Shorr modification of the Sumner method (27).

Enolase was assayed by following the appearance of phosphoenolpyruvate at 230 mμ according to the method of Westhead and Malmstrom (28). The cuvette contained, in a 3 ml final volume, 3 μmoles $MgCl_2$, 0.03 μmole EDTA, 7.5 μmoles 2-phosphoglycerate, 150 μmoles tris, pH 7.4, and 1.5 μmoles KCl, and 100,000 × *g* supernatant.

Condensing enzyme was measured by the method of Srere and Kosicki (26). The reaction mixture contained 70 μmoles tris, pH 8.0, 0.4 μmole oxaloacetate, 0.14 μmole acetyl-CoA and extract in a total volume of 1.0 ml.

Aconitase was measured by following the formation of *cis*-aconitate by the increase in absorption of 240 mμ. (3). The extract used was prepared in phosphate buffer.

Isocitric dehydrogenase was measured spectrophotometrically according to the method of Ochoa (17). The reaction mixture consisted of extract, 75 μmoles tris, pH 7.4, 1.8 μmoles $MnCl_2$, 0.135 μmole NADP, and 0.6 μmole DL-isocitrate in a volume of 3.0 ml.

α-Ketoglutaric dehydrogenase was assayed by following the formation of NADH at 340 mμ (12). The reaction mixture consisted of 0.13 μmole CoA, 100 μmoles potassium phosphate, pH 7.4, 0.27 μmole NAD, 0.83 μmole L-cysteine, 25 μmoles α-ketoglutarate, 30 μmoles $MgCl_2$, 0.3 μmole TPP, and extract in a total volume of 3.0 ml. The blank contained all of the components of the reaction mixture except CoA. The reaction was initiated by the addition of CoA, but could be initiated as well by the addition of α-ketoglutarate. No reduction of NAD was observed in the absence of TPP.

Succinyl-CoA synthetase was assayed by the method of Cha and Parks (6).

Succinate dehydrogenase was measured spectrophotometrically according to Massey (20). The cuvettes contained, in a final volume of 1 ml, 50 μmoles potassium phosphate, pH 7.4, 0.52 mg cytochrome C, 20 μmoles succinate, and 16.5 μg phenazine methosulfate.

Fumarase was measured by following the disappearance of fumarate at 300 mμ (19). The reaction mixture contained, in a volume of 3 ml, 100 μmoles potassium phosphate, pH 7.4, 50 μmoles potassium fumarate, and extract prepared in phosphate buffer.

Malate dehydrogenase was measured by following the oxidation of NADH at 340 (18). The reaction mixture contained, in a final volume of 3 ml, 100 μmoles tris, pH 7.4, 0.15 μmole NADH, and 0.3 μmole oxaloacetate.

Protein was measured by reaction with Biuret reagent (15).

ATP, NADH, NADP, NADPH, horse heart cytochrome C, TPP, fructose 1,6-diP (sodium salt), 3-phosphoglycerate (tricyclohexylammonia salt), the barium salts of ribulose 1,5-diP, 2-phosphoglycerate and phosphoenolpyruvate, DL-isocitric acid, α-ketoglutaric acid, fumaric acid, tris, and the enzymes used in coupled reactions were obtained from the Sigma Chemical Company; CoA, acetyl-CoA, and NAD from Pabst Laboratories; oxaloacetic, succinic and citric acids, L-cysteine, and GSH from Calbiochem, and the barium salt of ribose 5-P from C. F. Boehringer and Soehne. Sodium salts of phosphoenolpyruvate, ribulose 1,5-diP, and 2-phosphoglycerate were prepared by the addition of sodium sulfate to a solution of the respective barium salt in dilute HCl. The barium salt of ribose 5-P was dissolved in pH 7.0 tris buffer and Ba^{2+} precipitated as $BaSO_4$. L-Malate, used in growth media, was C grade, obtained from Calbiochem; sodium acetate (AR grade, Mallinkrodt) was recrystallized from water. All other chemicals used were analytical reagent grade.

Results

The levels of several of the enzymes of the reductive pentose phosphate cycle in *R. rubrum*, grown autotrophically, photoheterotrophically with L-malate or acetate as carbon sources, or under dark aerobic conditions, are given in table I.

Ribulose 1,5-diP carboxylase, phosphoribulokinase, and alkaline fructose 1,6-diPase levels are highest in autotrophically grown cells. The level of ribulose 1,5-diP carboxylase is to our knowledge, the highest which has been reported for any organism. Levels of these enzymes are significantly higher in the acetate grown organism than in the malate grown organism. The presence of light, per se, during growth has little effect on the levels of these enzymes, as is seen by inspection of the values obtained for light and dark grown malate heterotrophs.

The apparently lower levels of activity for glyceraldehyde 3-P dehydrogenase in cells cultured on L-malate may be a result of a difference in the affinity of the enzyme for the substrate (1,3-diphosphoglycerate) and not to actual differences in the real levels of the enzyme since the levels of substrate used in the assay were lower than those needed for optimal activity of the *Chromatium* enzyme as isolated from heterotrophically grown cells, but equal to the levels needed for optimal activity of the enzyme isolated from autotrophically cultured *Chromatium* (11). It is significant that the acetate cells resemble the autotrophs and that light has no affect on the enzyme in malate heterotrophs.

There was a 5-fold stimulation of aldolase activity by Fe^{2+}. The rate of the reaction was slightly (about 10 %) enhanced by the addition of EDTA (to 1mM) in the absence of iron.

The acetate grown cells contained a neutral phosphatase which was active towards fructose

Table I. *Levels of Enzymes of the Reductive Pentose Phosphate Cycle and EMP Pathway in* Rhodospirillum rubrum

Extracts prepared as described in Methods section. Specific activities, (μmoles substrate consumed min^{-1} g^{-1} protein), are based on protein in the crude extract.

	Conditions of growth			
	Autotroph	Acetate $(NH_4)_2SO_4$ phototroph	L-Malate $(NH_4)_2SO_4$ phototroph	L-Malate $(NH_4)_2SO_4$ dark heterotroph
Ribulose 1,5-diP carboxylase*	240	60	7	4
Ribulose 5-P kinase	34	33	12**	0**
Ribose 5-P isomerase	40	77	51	66
Glyceraldehyde 3-P dehydrogenase	49	36	11	12
Aldolase	9.4	7.6	9.2	11
Fructose 1,6-diPase (neutral)	0	18	2	2
Fructose 1,6-diPase (alkaline)	27	18	1	1
Enolase	80	110	96	140

* μMoles of phosphoglycerate formed.
** ATPase interferes.

Table II. *Levels of Enzymes of the Tricarboxylic Acid Cycle in* Rhodospirillum rubrum

Extracts prepared as described in Methods section. Enzyme levels are expressed as μmoles min^{-1} g^{-1} protein.

	Conditions of growth			
	Autotroph	Acetate $(NH_4)_2SO_4$ phototroph	L-Malate glutamate phototroph	L-Malate $(NH_4)_2SO_4$ phototroph	L-Malate $(NH_4)_2SO_4$ dark heterotroph
Condensing enzyme	140	190	105	35	140
Aconitase	110	100	150	110	170
Isocitric dehydrogenase	190	220	170	225	459
α-Ketoglutaric dehydrogenase	4.1	2.2	6.6	6.6	5.6
Succinyl-CoA synthetase	105	88	60	66	96
Succinic dehydrogenase	1560	2180	2230	1510	2220
Fumarase	580	124	420	510	800
Malic dehydrogenase	1900	3700	2460	1390	2350

1,6-diP. This activity was not found in this organism cultured under any other set of conditions.

In table II the activity of the tricarboxylic acid cycle enzymes are listed as measured in extracts of cultures grown under a variety of conditions including conditions such that hydrogen is evolved (with glutamate as a nitrogen source) and is not evolved (with ammonium sulfate as nitrogen source) during growth.

The levels of the tricarboxylic acid cycle enzymes are somewhat higher in dark than in light grown malate heterotrophs. This probably reflects the fact that a large percentage of the protein of the phototroph is in the chromatophore. Condensing enzyme is markedly lower in the malate photoheterotroph.

The condensing enzyme is likewise markedly lower in the non-hydrogen producing malate photoheterotroph than in the organism from cultures of malate photoheterotrophs photoevolving hydrogen. Likewise malate dehydrogenase was higher in the hydrogen evolving organism. The fumarase from cultures of glutamate, malate photoheterotrophs required the addition of a thiol to the extraction media for stability. 20 mM Mercaptoethanol was used here[2]. This fumarase activity was lost when the extract was prepared in tris buffer. The fumarase activity of the malate, ammonium sulfate photoheterotrophs was not affected by thiols, nor was it affected by tris.

The condensing enzyme and malic dehydrogenase levels are high in the acetate photoheterotroph; fumarase levels are low.

Discussion

Clearly the enzyme profile with regard to photosynthetic carbon metabolism of autotrophically grown *R. rubrum* is similar to that of higher green plants and algae. The results of these experiments and the kinetics of CO_2 fixation as reported in the previous paper are consistent with the operation of the reductive pentose phosphate cycle, or of a modification thereof, for the photometabolism of CO_2.

[2] Thiol stabilization of a bacterial fumarase was first observed by Dr. A. G. Callely in this laboratory with extracts of a *Chloropseudomonad*.

Under autotrophic conditions ribulose 1,5-diP carboxylase, ribulose 5-P kinase, alkaline fructose 1,6-diPase, and high levels of glyceraldehyde 3-P dehydrogenase activity are induced. Light, per se, had no effect on the induction of these enzymes since the levels in cells grown either in darkness or in the light on L-malate, ammonium sulfate media were similar. It would seem rather that the carbon source is more directly involved with control than is light.

Ribulose 1,5-diP carboxylase levels have been shown to be a function of the mode of growth in a number of photosynthetic and chemosynthetic forms (9, 13, 16, 24). In *Rhodopseudomonas spheroides* Lascelles found that ribulose 1,5-diP carboxylase activity was light induced (14). This is in contrast to the results obtained here. Ribulose 1,5-diP carboxylase levels in autotrophically cultured *R. spheroides* (L. Anderson, unpublished results) are similar to those obtained by Lascelles in the photoheterotrophically cultured organism. It would appear that the difference in the effect of light on the induction of this enzyme may be a species difference; possibly malate represses formation of this enzyme in *R. rubrum* and *R. spheroides* is not subject to this repression.

Ribulose 5-P kinase was found to be higher in autotrophically cultured than in heterotrophically cultured *Chlamydomonas mundana* by Russel and Gibbs (24). Similar results were found in the present experiments. These authors found that ribose 5-P isomerase levels were much higher in autotrophic *C. mundana*. In *R. rubrum*, in contrast, levels of the latter enzyme are lowest in the autotrophically cultured organism and highest in the acetate photoheterotroph; in any case the levels of this enzyme are high enough to account for photosynthesis. This enzyme is probably not under metabolic control in *R. rubrum*.

Fructose 1,6-diP aldolase in this organism appeared in metal ion specificity to be similar to that found in *R. spheroides* (29), *Anacystis nidulans* (29), and in heterotrophically cultured *Chlamydomonas* (24). It does not appear to be subject to metabolic control related to photosynthesis.

The level of glyceraldehyde 3-P dehydrogenase, as measured in these experiments, is highest in the autotrophs and in the acetate photoheterotrophs. The levels of activity in both the light and in dark grown malate heterotrophs are 4-fold lower than in the autotrophs. Clearly this enzyme is subject to a form of metabolic control related to photosynthetic carbon metabolism. Similar results have been obtained with *Chromatium* (11). In green plants the nucleotide specificity of this enzyme is under comparable control (8).

Alkaline fructose 1,6-diP phosphatase in *R. rubrum* appears to be induced under conditions of autotrophy. This enzyme has been shown to be associated with the chloroplast in green plants and to be light induced in the algae, *Euglena* (25). Apparently in *R. rubrum*, this enzyme is induced (or de-repressed), not by light, but by autotrophic growth conditions.

The photometabolism of CO_2 by acetate photoheterotrophs appeared to be intermediate between strict photoheterotrophy as exemplified by the malate photoheterotrophs and strict autotrophy (2). With regard to the photosynthetic enzymes, the enzyme profile presented here for the acetate photoheterotroph is intermediate between that of the strict heterotrophs (malate cultures) and the autotrophs. In every instance where a photosynthetic enzyme level was high in the autotroph it was higher in the acetate photoheterotroph than in the malate photoheterotroph. It would appear that this organism, when grown phototrophically on acetate, can utilize CO_2 photosynthetically, thus circumventing the necessity for isocitritase and the glyoxylate cycle.

It is apparent that light, alone, is not responsible for the appearance of the enzymes of the reductive pentose phosphate cycle in this organism. Cohen-Basire and Kunisawa (7) have shown that light is not responsible for the induction of the enzymes of photophosphorylation or for the formation of the photochemical apparatus of this organism. Clearly, any effect of light on photosynthetic carbon metabolism in this bacterium is a secondary effect, related to the changes in levels of adenine nucleotides and possibly reduced pyridine nucleotides.

Enolase activity was essentially the same regardless of the mode of growth. It has been postulated that the enzymes of the Embden-Meyerhof pathway (from glyceraldehyde 3-P dehydrogenase through enolase) are under the control of a single genetic system (22). If this is true in *R. rubrum* then the relative activity of this pathway exclusive of glyceraldehyde 3-P dehydrogenase is unaffected by light or by the carbon or nitrogen source used for growth.

All of the enzymes of the tricarboxylic acid cycle were found in *R. rubrum* grown under the conditions tested. The condensing enzyme appears to be under metabolic control in this organism, as does isocitric dehydrogenase, malic dehydrogenase and possibly α-ketoglutaric dehydrogenase. The present experiments are not sufficiently detailed to allow speculation as to the details of the control of these 3 enzymes; O_2 tension probably influences the first 3 mentioned enzyme levels since they are significantly higher in L-malate, ammonium sulfate cultures grown aerobically than phototrophically.

Fumarase in extracts of the hydrogen evolving photoheterotroph appears to have quite different properties from the fumarase in extracts prepared from non-hydrogen evolving forms. This enzyme has been shown to be under allosteric control in several organisms (4). It is tempting to correlate the requirement for thiol with the ability of the cell to produce hydrogen. It is clear from these experiments that the photoevolution of hydrogen is not controlled by simple adaptive formation of 1 or more enzymes of the tricarboxylic acid cycle, since light has little or no effect on the levels of the tricarboxylic acid cycle enzymes.

Metabolic control in this organism appears to be mediated entirely by the nature of the carbon and nitrogen sources utilized for growth. Light had no demonstrable effect on the presence or levels of any of the enzymes of photosynthetic and heterotrophic carbon metabolism studied.

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