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Regulation of Glucose 6-Phosphate Dehydrogenase in Blue-Green Algae[1]

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ABSTRACT

Glucose 6-phosphate dehydrogenase (EC 1.1.1.49) has been partially purified from *Anacystis nidulans* and *Anabaena flos-aquae* by means of ammonium sulfate fractionation and exclusion gel chromatography and the kinetic properties determined.

Glucose 6-phosphate dehydrogenase from these blue-green algae exhibits Michaelis-Menten kinetics at pH 6.7. At this pH, Km values of 0.37 mM for glucose 6-phosphate and 10 μM for NADP were determined. At a pH above 7.4, the enzyme exhibits sigmoidal kinetics with respect to glucose 6-phosphate saturation but the saturation curve for NADP remains hyperbolic.

ATP is an inhibitor of the enzyme competitively with NADP with a Ki of 2 to 5 mM. NADPH inhibits the enzyme competitively with glucose 6-phosphate. The inhibition curves for NADPH are hyperbolic at pH 6.7 and sigmoidal at pH 8.6.

The significance of these *in vitro* kinetics are discussed relative to *in vivo* data on the control of glucose 6-phosphate turnover in blue-green algae.

The blue-green algae are procaryotic organisms with a generally obligate photoautotrophic mode of growth (22). Their mechanism for fixing CO_2 seems to be similar to the mechanism exhibited by higher plants, but they display several metabolic irregularities (14). These organisms do not appear to have a complete Krebs cycle: the presence of α-ketoglutarate dehydrogenase and succinyl CoA synthetase could not be detected in a variety of blue-green algae (15, 21). In addition, glycolysis appears to be blocked due to a low level or absence of phosphofructokinase (16; G. Kelly and R. E. McGowan, unpublished results). Thus, the two catabolic pathways for carbon flow present in most organisms show restricted activity in the cyanophyta. Using radioactive glucose, Cheung and Gibbs (3) found that catabolism in *Tolypothrix* occurred via the pentose shunt. A catabolic role for this pathway in blue-green algae has also been suggested by Pelroy and Bassham (17). The relationship between the degradative shunt and photosynthesis has come under scrutiny only recently. It has been suggested that the pentose phosphate pathway may be regulated by photosynthesis, and that this regulation may be expressed at the first enzyme of the pathway, glucose-6-P dehydrogenase (16).

Pelroy and Bassham (17) have found that photosynthesis causes an increase in the glucose-6-P pool size. In view of the finding that the pool size of 6-P-gluconate is very small during photosynthesis, it has been suggested that the blockage occurs at the point of conversion of glucose-6-P to 6-P-gluconate (16, 17). Such data indicate that photosynthesis may regulate the flow of carbon through the pentose phosphate pathway by controlling the activity of glucose-6-P dehydrogenase. A variety of factors may contribute to the apparent light-promoted decrease in dehydrogenase activity. The enzyme isolated from the sweet potato tuber displays decreased activity in the presence of either NADPH or ATP (11). During photosynthesis, noncyclic electron transport results in an increased cellular NADPH concentration, whereas cyclic electron flow causes an increase in the cellular ATP concentration. Such increases may reduce the rate of carbon flow through the pentose phosphate pathway via suppression of glucose-6-P dehydrogenase activity. It has recently been reported that ribulose-1,5-diP, a Calvin cycle intermediate which also shows a marked increase during photosynthesis, causes diminished dehydrogenase activity (16). In addition to the possible enzyme regulation via Calvin cycle intermediates and electron flow products, light-induced pH changes in the extrathylakoidal environment may cause an alteration in enzyme activity. Neumann and Jagendorf (13) have demonstrated that light causes an uptake of hydrogen ions by the membranous thylakoid particles found in chloroplasts. This proton uptake would result in an increased pH outside the thylakoid. In this study of glucose-6-P dehydrogenase, the enzyme's kinetic parameters are shown to be markedly different at different pH values. In fact, pH changes can transform the kinetics of glucose-6-P dehydrogenase from the typical Michaelis-Menten type to the sigmoidal type. Kinetic comparisons among the glucose-6-P dehydrogenases from several organisms indicate that the kinetic transformation may be unique to blue-green algae.

MATERIALS AND METHODS

Chemicals. Glucose-6-P monosodium salt, 6-P-gluconate trisodium salt, glucose-1-P dipotassium salt (glucose-1,6-diP free), glucose-1,6-diP tetracyclohexylammonium salt, ribose-5-P disodium salt, ribulose-1,5-diP dibarium salt, ATP disodium salt, NADP sodium salt, NADPH tetrasodium salt, and NAD were purchased from Sigma. The glucose-6-P was chromatographically pure; however, the ribulose-1,5-diP con-

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tained at least 50% glycerate-3-P. All other chemicals were reagent grade.

Growth of Organisms. *Anacystis nidulans* (Bloomington 625) and *Anabaena flosa-aquae* (Bloomington 1444) were obtained from the Indiana Algal Culture Collection and grown at 25 C in modified Bristol's solution plus soil extract (23) with constant agitation on a gyratory shaker. Illumination (3.5×10^5 ergs cm^{-2} sec^{-1} at the shaker surface) was continuously provided by six 20-w Sylvania Gro-Lux lamps placed 54 cm above the surface of the shaker. Carboy cultures of 8 liters were maintained with constant illumination at the carboy midpoint of 6×10^5 ergs cm^{-1} provided by two banks of 40-w fluorescent bulbs. Agitation was by constant bubbling of sterile air through the carboy.

Rhodospirillum rubrum was grown in stationary cultures in the medium of Newton (12) and provided with constant illumination of 3×10^5 ergs cm^{-2} sec^{-1} at the flask base. *Pisum sativum* var. Progress No. 9 was grown in vermiculite in a glass house for 2 weeks prior to harvesting. *Euglena gracilis* var. bacillaris, grown mixotrophically with constant agitation on a gyratory shaker in Difco *Euglena* medium, was kindly provided by Dr. Melvyn Selsky.

Enzyme Preparation. The blue-green algae and bacteria were frozen as a cell suspension at −77C in the chamber of an Eaton press (5), and the frozen cells were extruded through the orifice at 20,000 p.s.i. The broken cells were thawed and then centrifuged at 4 C for 10 min at 10,000*g*. The supernatant solution from the above centrifugation was fractionated between 20 and 37% saturation with ammonium sulfate by addition of solid. The 20 to 37% ammonium sulfate precipitate was redissolved in 0.1 M tris-0.1 M maleate buffer, pH 6.7, and dialyzed overnight against the same buffer at 4 C. Further purification was achieved by passing the protein solution through Sephadex G-200 or Sepharose 6B equilibrated with 0.1 M tris-0.1 M maleate, pH 6.7.

The pea shoots were homogenized in a Waring Blendor with 1:1 (w/v) 0.1 M tris-0.1 M maleate pH 6.7, filtered through several layers of cheesecloth, and centrifuged at 4 C for 10 min at 10,000*g*. The supernatant solution from this centrifugation was further purified in the same manner as the enzyme from blue-green algae.

Assays. Routine assays for glucose-6-P dehydrogenase were performed using either 1- or 3-ml assays containing 50 μmoles of tris; 50 μmoles of maleate, pH 6.7, 0.2 μmole of NADP, and 33 μmoles of glucose-6-P per ml assay mixture. Reactions were initiated by the addition of enzyme and were linear for 10 min under conditions of saturating substrate.

In the tests for phosphatase activity, enzyme extract was incubated at pH 6.7 with glucose-6-P, and Pi was determined by the method of Fiske and SubbaRow (6).

The assay for 6-P-gluconate dehydrogenase contained 0.2 μmole of NADP, 50 μmoles of tris, 50 μmoles of maleate, pH 6.7, and 7.5 μmoles of 6-P-gluconate in a total volume of 3 ml.

Phosphoglucomutase activity was assayed by coupling the reaction to the glucose-6-P dehydrogenase present. The 3-ml reaction mixture contained 0.2 μmole of NADP, 50 μmoles of maleate, pH 7, 7.5 μmoles of glucose-1-P, and 1 μmole of glucose-1,6-diP.

Phosphoglucoisomerase activity was also assayed by coupling to the glucose-6-P dehydrogenase present. The reaction mixture was the same as for phosphoglucomutase, except that fructose-6-P was substituted for the glucose-1-P.

In all of the assays, except the Pi determination, activity was measured as reduction of NADP monitored by an increase in absorbance at 340 nm using a Gilford 240 spectrophotometer equipped with a Sargent-Welch SRG recorder.

RESULTS

After ammonium sulfate fractionation, dialysis, and Sephadex G-200 column chromatography, no interfering enzyme activities were apparent in the glucose-6-P dehydrogenase preparations. Phosphoglucomutase, phosphatase, phosphoglucoisomerase, and 6-P-gluconate dehydrogenase activities could not be detected in the enzyme solutions, and the preparations were not able to reoxidize NADPH. Using this procedure, the enzyme was purified approximately 15-fold.

Substrate saturation curves for glucose-6-P dehydrogenase from *Anacystis nidulans* at a variety of pH values are shown in Figure 1. A hyperbolic substrate saturation curve is obtained at pH 6.7; from a double reciprocal plot of these data, a *Km* of 0.37 mM was determined. As the pH of the reaction mixture is increased, an increasing tendency towards a sigmoidal substrate saturation curve becomes apparent. At pH 8.4 an $S_{0.5}$ of 1.5 mM was determined (9). A Hill plot of these data results in a slope of $n = 2.78$, indicating a high degree of positive cooperativity (9). Essentially, the same results are obtained with the enzyme from a quite different blue-green alga, *Anabaena flos-aquae,* which is a member of the *Nostocaceae* (Fig. 2). There are some quantitative differences between the enzymes from *Anacystis* and *Anabaena.* At the lower pH, the enzyme from *Anabaena* has a *Km* for glucose-6-P of 0.15 mM. The shift towards sigmoidal kinetics with respect to glucose-6-P saturation occurs at a lower pH for the enzyme from *Anabaena* than for the enzyme from *Anacystis.*

Glucose-6-P dehydrogenase has been studied with a variety of organisms (7, 8, 10, 18, 20, 24) and has been found to be regulatory in several of them (7, 10, 18). Due to the important branch point position of the enzyme with respect to carbon flow in blue-green algae, it was important to know whether or not these sigmoidal kinetics were typical of other photosynthetic organisms. Glucose-6-P saturation curves for the enzyme from *Euglena gracilis* are presented in Figure 3. While pH does alter the maximum velocity of the reaction catalyzed by the enzyme from this organism, there is no apparent change in the affinity of this enzyme for glucose-6-P with changing pH.

Similar results were obtained with the glucose-6-P dehy-

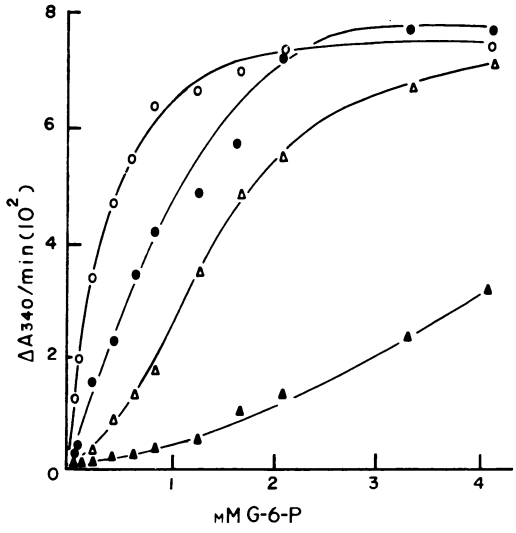

FIG. 1. Substrate saturation curves for glucose-6-P dehydrogenase from *Anacystis nidulans* at several pH values. The buffer, 0.1 M tris-0.1 M maleic acid, was adjusted to the desired pH. Assays at pH 6.7 (○), pH 7.4 (●), pH 7.8 (△), and pH 8.6 (▲).

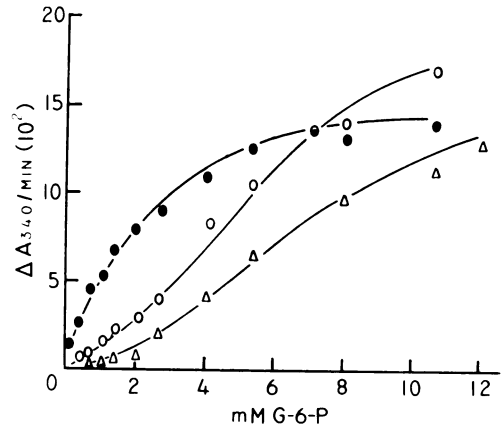

FIG. 2. Substrate saturation curves for glucose-6-P dehydrogenase from *Anabaena flos-aquae* at several pH values. The buffer, 0.1 M tris-0.1 M maleic acid, was adjusted to the desired pH. pH 6.7 (●), pH 7.4 (○), and pH 8.6 (△).

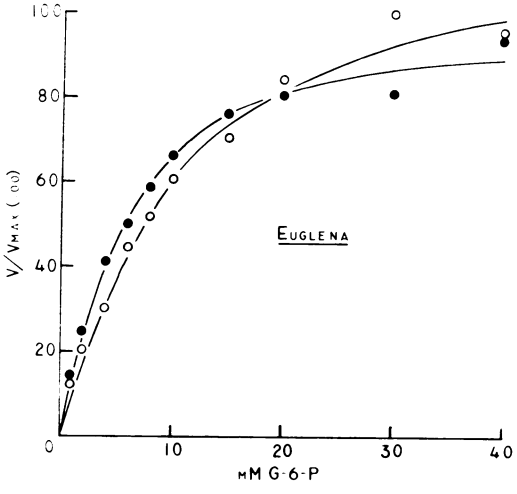

FIG. 3. Substrate saturation curves for glucose-6-P dehydrogenase, *Euglena gracilis*, at pH 6.7 (○) and pH 8.6 (●).

drogenases from *Rhodospirillum rubrum* and *Pisum sativum*. In the case of *Pisum* no attempt was made to separate the chloroplastic from the cytoplasmic enzymes (1).

NADP, the coenzyme for glucose-6-P dehydrogenase, is not involved in the regulatory kinetics of the enzyme. The NADP saturation curves at pH 8.6 and 6.7, at saturating glucose-6-P for these two pH values, are hyperbolic and give *Km* values in the region of 10 μM NADP. Varying the glucose-6-P concentration at either pH does not alter the shape of the NADP saturation curve or the apparent *Km* values. Also, the shape of the glucose-6-P saturation curves at the various pH values was independent of NADP concentration. These data suggest that the sigmoidal kinetics associated with glucose-6-P in slightly basic conditions are due to homotropic interactions, and that pH is the modifier.

Several attempts that were made to alter the shape of the pH 8.6 or pH 6.7 glucose-6-P saturation curves proved unsuccessful. Fructose-6-P, ribose-5-P, glucose-1-P, ribulose-5-P, glyceraldehyde-3-P, 3-P-glyceric acid, and 6-P-gluconic acid all were without effect. Ribulose-1,5-diP, which has been reported by Pelroy *et al.* (16) to be inhibitory to the enzymes from *Aphanocapsa* and *Synecoccus*, was without effect in our experiments between 0.5 and 10 mM. At present we cannot explain this disparity. Neither pH, glucose-6-P, or NADP concentrations promote ribulose-1,5-diP inhibition.

It was found that two products of the light reactions of photosynthesis, NADPH and ATP, have an inhibitory effect. ATP is a competitive inhibitor of NADP and a noncompetitive inhibitor of glucose-6-P (Fig. 4). The inhibition curves follow Michaelis-Menten kinetics at both pH 6.7 and 8.6. From the data in Figure 4, the K*i* for ATP is between 2 and 5 mM.

NADPH does not exhibit as simple a picture of inhibition as ATP. Figure 5 shows two Dixon plots which suggest that at pH 6.7 NADPH inhibits glucose-6-P dehydrogenase competitively with glucose-6-P, and noncompetitively with NADP with a K*i* of 0.13 mM. These results are somewhat surprising considering that NADPH is also a product of the glucose-6-P dehydrogenase reaction, and it might be expected that the product inhibition is competitive with NADP. At pH 8.6 the NADPH inhibition studies reveal very different inhibition kinetics (Fig. 6). At this pH, the shape of the inhibition curve is sigmoidal regardless of whether NADP or glucose-6-P is varied. The results of such inhibition are evidenced by the nonlinearity of the Dixon plots. The apparent sigmoidal binding of NADPH to the enzyme at pH 8.6 is consistent with its competition with glucose-6-P and the sigmoidal binding of glucose-6-P to the enzyme at pH 8.6.

Anderson *et al.* (1) have investigated the effect of dithiothreitol on the glucose-6-P dehydrogenases from *Pisum* and have found this compound to inhibit the enzymes. In our studies on the enzyme from blue-green algae, sulfhydryl reagents and thiols were tested for their effect on the catalytic rate. Our results indicate that hydroxymercuribenzoate (K*i* 0.3 μM) and N-ethyl maleimide (K*i* 200 μM) are potent in-

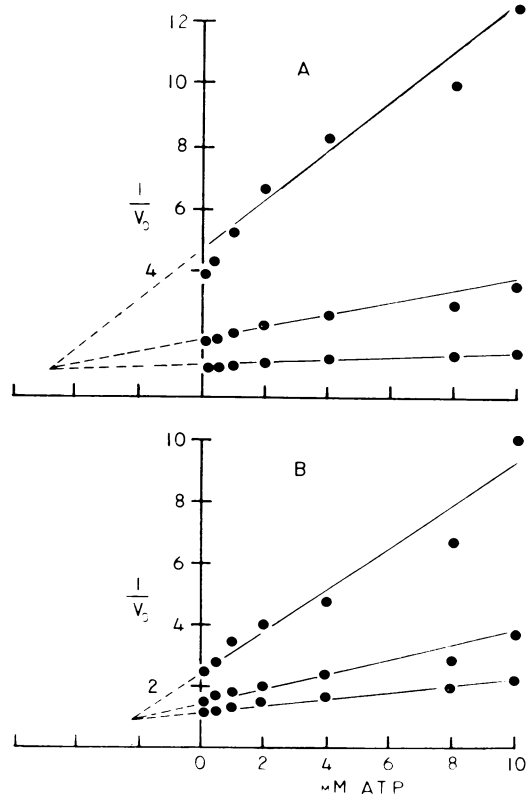

FIG. 4. A Dixon plot of the effect of ATP on glucose-6-P dehydrogenase from *Anacystis nidulans* at several NADP concentrations. A: pH of the assay was 6.7 and the NADP concentrations were 10, 30, and 100 μM respectively. B: pH of the assay was 8.6 and the NADP concentrations were 10, 30, and 100 μM respectively.

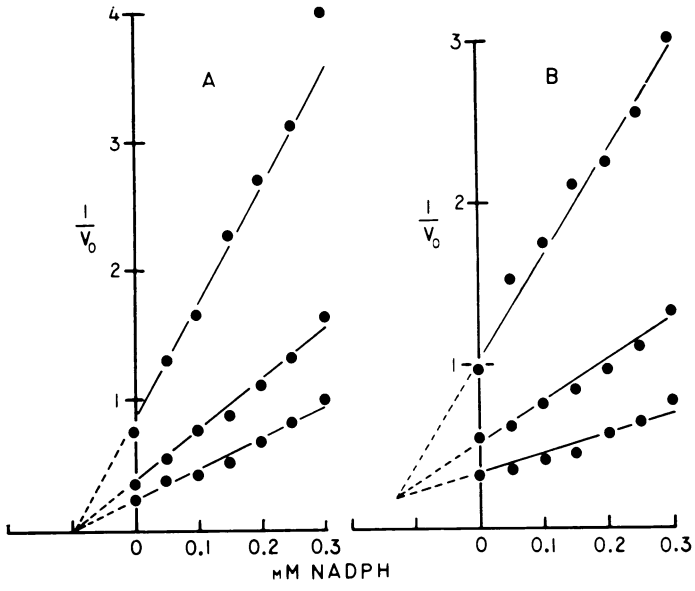

FIG. 5. A Dixon plot of the effect, at pH 6.7, of NADPH on glucose-6-P dehydrogenase from *Anacystis nidulans* at several NADP concentrations (A) and at several glucose 6-P concentrations (B). A: NADP concentrations were 10, 30 and 100 μM, respectively; B: glucose-6-P concentrations were 0.1, 0.3, and 1 mM, respectively.

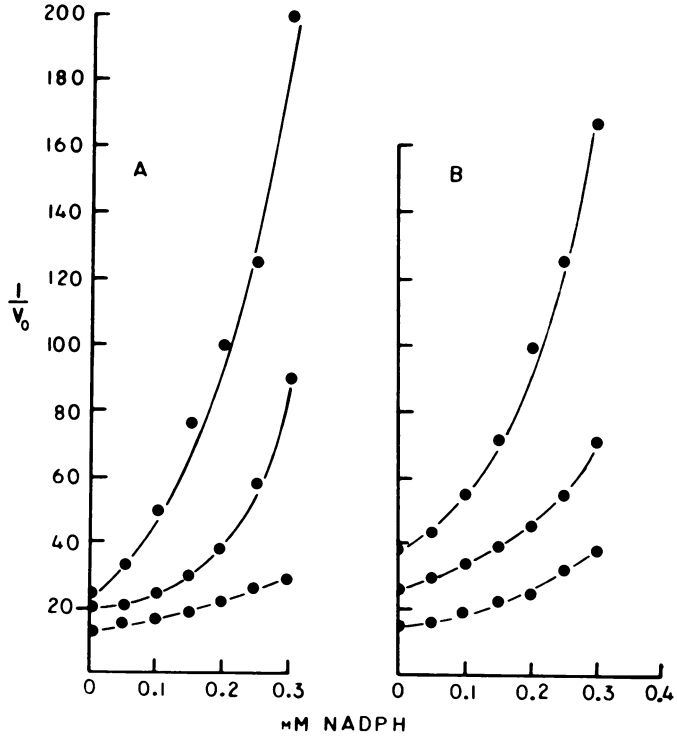

FIG. 6. A Dixon plot of the effect, at pH 8.6, of NADPH on glucose-6-P dehydrogenase from *Anacystis nidulans* at several NADP concentrations (A) and at several glucose 6-P concentrations (B). A: NADP concentrations were 10, 30, and 100 μM, respectively; B: glucose-6-P concentrations were 0.1, 0.3, and 1 μM, respectively.

hibitors of the enzyme and that iodoacetic acid is not. These results suggest that either a sulfhydryl group is present at the active site or is important in maintaining an active conformation. Dithiothreitol and β-mercaptoethanol had little or no effect on the enzyme from either *Anacystis* or *Anabaena*, unlike the enzyme from *Pisum*.

DISCUSSION

A variety of enzymes have been found to exhibit regulatory kinetics. Recently it has been reported that sweet potato glucose-6-P dehydrogenase saturation curves deviate from typical hyperbolic curves (10). Enzymes having more than one catalytic site are thought to deviate from Michaelis-Menten kinetics as a result of ligand-induced conformational changes (9). Initial substrate attachment may result in an increased or decreased enzyme affinity for substrate at secondary sites. The regulatory kinetics of glucose-6-P dehydrogenase in blue-green algae has particular significance because of the reported sugar phosphate labeling patterns exhibited by these organisms during light-dark transitions (17). In the light, the algae accumulate radioactivity from $^{14}CO_2$ into glucose-6-P, while the amount of radioactivity in 6-P-gluconate is negligible. During the ensuing dark period, the situation is reversed. The amount of radioactivity in glucose-6-P diminishes with a concomitant increase in the amount of radioactivity associated with 6-P-gluconate. Pelroy *et al.* (16) have shown that glucose turnover is limited in the light, and increased in darkness or in the presence of DCMU. In this laboratory, similar results have been obtained on the rate of turnover of the C-1 carbon of glucose-6-P in *Anabaena* (Rubin and McGowan, unpublished results). These two studies point to a fine control at the site of the glucose-6-P reaction in which some product(s) of photosynthesis feed back to control the entrance of glucose-6-P into the pentose phosphate pathway. The necessity for this type of control is logical considering the negligible phosphofructokinase activity in these organisms (17), the catabolic functioning of the pentose-P pathway (3), and the lack of a chloroplast membrane to control Calvin cycle pool sizes.

Pelroy *et al.* (16) have implicated ribulose-1,5-diP as a control compound for glucose-6-P dehydrogenase activity. Although we cannot confirm their results, it does not necessarily negate ribulose-1,5-diP as a possible control compound. One thing is certain: other products of photosynthesis also influence the catalytic activity of this enzyme. The pool sizes of certain Calvin cycle intermediates increase, and the other influential products of photosynthesis are (the reductant) NADPH, (the high-energy phosphate) ATP, and a pH gradient across the thylakoid membrane (13). We have shown that both ATP and NADPH are inhibitors of the enzyme from blue-green algae. The nature of this inhibition differs from that of the enzyme from the nonphotosynthetic tissue of the sweet potato tuber (11). We have also shown that pH has a significant effect on the kinetics of the enzyme. If glucose-6-P dehydrogenase is in some proximity to the thylakoids of these prokaryotes, it is then reasonable to assume that the extrathylakoidal alkalinity caused by proton uptake into the thylakoid could decrease the enzyme's affinity for substrate and appreciably inhibit the turnover of glucose-6-P. The occurrence of such a pH gradient in blue-green algae has been demonstrated (19). The magnitude of these pH shifts *in vivo* is speculative, but if the enzyme is localized in this changing environment, then its catalytic activity will be influenced.

Another question is, what are the *in vivo* levels of NADPH and ATP in these organisms during steady state photosynthesis? Biggins (2), investigating the respiratory capacity of *Anacystis nidulans* under aerobic and anaerobic conditions, determined the equilibrium pool sizes of ATP, ADP, AMP,

NADP, NAD, NADPH, and NADH. From his results, we calculate that during photosynthesis the ATP concentrations in these cells would be in the range of 1 to 2 mM, and the NADPH concentration would be in the range of 100 to 500 μM. These NADPH concentrations would, according to our kinetic experiments, have significant effects on glucose-6-P dehydrogenase catalysis. The ATP concentrations are in the range of the K*i* for ATP and can also be considered to have a significant regulatory role for this enzyme.

In summary, we have presented evidence which, although insufficient to explain entirely obligate photoautotrophy in blue-green algae, does explain the observed metabolic control at the first reaction of the oxidative pentose phosphate pathway.

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