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Possible Mechanism of Mannose Inhibition of Sucrose-Supported Growth in N_2-Fixing *Azotobacter vinelandii*

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When mannose was added to a sucrose-supported culture of *Azotobacter vinelandii* under N_2-fixing conditions, cell growth was inhibited. The degree of inhibition was proportional to the amount of mannose and to the aeration rate (T.-Y. Wong, Appl. Environ. Microbiol. 54:473–475, 1988). In this report, we demonstrate that once inside the cell, mannose was phosphorylated to mannose 6-phosphate. It was then isomerized to fructose 6-phosphate and to glucose 6-phosphate. Mannose inhibited sucrose uptake noncompetitively. The decrease in sucrose uptake after mannose addition coincided with a lower rate of respiration and a decrease in nitrogenase activity. The decrease in sucrose uptake and in the ATP pool may decrease the electron flow and reduce protection of the nitrogenase from O_2. Cells became very sensitive to O_2, and therefore, cell growth was inhibited under high aeration conditions.

Azotobacter vinelandii is a free-living N_2-fixing organism. This obligate aerobe generates its ATP by oxidative phosphorylation. This organism also has a very high rate of respiration. In addition to the normal energy needed for metabolism, the high rate of respiration provides *A. vinelandii* with reducing power for both N_2 fixation and protection of the nitrogenase from O_2 under aerobic conditions (14). *A. vinelandii* can grow on a wide variety of organic substances under N_2-fixing conditions (19). *A. vinelandii* transports its carbohydrates by active transport (1, 3, 15). Once inside the cell, carbohydrates are used via the Entner-Doudoroff pathway (10, 11, 16). *A. vinelandii* exhibits diauxie when grown on a mixture of acetate and glucose. Acetate is used as the primary carbon source during acetate-glucose growth. Induction of the glucose transport system is repressed in the presence of acetate (4). Wong and Maier (19) showed that *A. vinelandii* cannot grow with mannose as a carbon and energy source under N_2-fixing conditions. However, *A. vinelandii* can grow mixotrophically on mannose with H_2 gas as an energy source. Other studies from the laboratory of Maier show that H_2 increases the V_{max} of mannose transport (9). In an extension of these studies, it was shown that mannose inhibits *A. vinelandii* growth under N_2-fixing conditions on sucrose and turanose media (17). This type of inhibition is specific for sucrose and turanose. The degree of inhibition by these sugars is influenced by the aeration rate.

The results described here show that mannose was phosphorylated and could eventually be converted to glucose 6-P inside the cell. However, the phosphomannose isomerase activity was very low. We also showed that mannose inhibited sucrose uptake noncompetitively in *A. vinelandii*. We hypothesized that the slowdown in nutrient supply reduced the electron flow. This slowdown was possibly the main cause of growth inhibition under highly aerated N_2-fixing conditions.

MATERIALS AND METHODS

Chemicals. Mannose, sucrose and [U-^{14}C]sucrose (specific activity, 80 mCi/mmol in 90% ethanol), NAD, glucose dehydrogenase, phosphomannose isomerase, phosphoglucose isomerase, mannose 6-phosphate, fructose 6-phosphate, and glucose 6-phosphate were purchased from Sigma Chemical Co., St. Louis, Mo. Mannose was further purified as described previously (17). [U-^{14}C]mannose (specific activity, 232 mCi/mmol in 20% ethanol) was purchased from ICN Pharmaceuticals, Inc. (Costa Mesa, Calif.). All other chemicals were of reagent grade and were purchased from J. T. Baker Chemical Co., Phillipsburg, N.J.

Cell growth. *A. vinelandii* OP (obtained from P. E. Bishop, Raleigh, N.C.) was grown under carbon-limiting, N_2-fixing conditions in Burk medium containing 0.5% sucrose as previously described (5, 18). Unless specified, all cultures were grown in 250-ml sidearm flasks containing 50 ml of medium. Cultures were incubated in an incubator shaker rotating at 300 rpm at 30°C. Growth was monitored at 560 nm with a Spec 20 spectrophotometer. An optical density unit corresponded to about 2.7×10^8 cells per ml.

Sugar uptake. A modified sugar transport procedure (9) was used to analyze the transport kinetics of sucrose and mannose. Cells were grown in Burk medium, harvested at mid-log phase, and washed three times with carbon-free Burk medium. Cells were suspended in carbon-free Burk medium to about 9×10^8 cells per ml. The culture was incubated at 30°C for 1 h with gentle stirring to deplete endogenous substrate. A 24-well tissue culture plate (Costar, Cambridge, Mass.) was used for the transport experiments. Each duplicate well contained 0.8 ml of Burk medium with isotopic sucrose (specific activity, 0.1 μCi/μmol; concentration range, 0.123 to 3 mM). Mannose (final concentration of 50 mM) was added to one set of wells. The plate was tapped on a commercial vibrating device (a Pollenex Deep Heat Massager worked fine) to provide instant mixing and aeration. The transport experiment was started by adding 0.2 ml of cell suspension to each well. After 4, 8, and 12 min, a 0.3-ml cell sample from each well was transferred to 1 ml of ice-cold 2% sucrose–Burk medium in a microcentrifuge tube. Cells were washed twice by centrifugation in a microcentrifuge with a vertical rotor for 30 s with ice-cold 2% sucrose–Burk medium. Cells were then dissolved in 1 ml of Ecolite (ICN). Samples were counted in a Beckman LS 7000 scintillation counter. Under the above conditions, the rate of sucrose uptake was linear for at least 12 min. Uptake rates presented in the figures are the means calculated from the six time points of the duplicates. The uptake rate was expressed as nanomoles of sugar taken up per minute per 10^9 cells.

Boiled cells were used to determine the background counts for nonspecific binding.

Isotopic mannose transport was done similarly, with mannose in place of sucrose. The specific activity of the isotopic mannose was 0.07 μCi/μmol. The concentration range of mannose used was from 0.12 to 15.2 mM.

Enzymatic analyses. The ability to phosphorylate mannose to mannose 6-P and subsequently convert it to glucose 6-P inside the cells was tested. A cytoplasmic crude extract fraction from sucrose-grown cells was prepared by differential centrifugation as previously described (5). The amount of mannose kinase was determined by measuring the reduction of NAD in a reaction mixture containing 50 mM Tris hydrochloride (pH 7.6), mannose (10 mmol), NAD (2 μmol), $MgCl_2$ (1 mM), phosphomannose isomerase (5 U), phosphoglucose isomerase (10 U), and glucose 6-P dehydrogenase (20 U). The final volume in the cuvette was 1 ml. The reaction was started by the addition of 20 to 50 μg of crude extract. The production of NADH was monitored spectrophotometrically at 340 nm. Phosphomannose isomerase was assayed similarly by adding mannose 6-P (1 mM) to the same incubation mixture without added phosphomannose isomerase or mannose. Phosphoglucose isomerase was assayed by incubating fructose 6-P (1 mM) in the reaction mixture without added mannose, phosphomannose isomerase, or phosphoglucose isomerase.

Mutant isolation. A spontaneous mutant resistant to mannose inhibition was isolated by growing *A. vinelandii* in 50 ml of Burk medium containing 0.5% sucrose plus 0.2% mannose. Once the culture reached stationary phase (5 to 7 days), 1 ml of cells was transferred to fresh Burk medium of the same composition. After about 4 months of being transferred, the culture reached stationary phase 2 days after inoculation. Cells from that flask were streaked on Burk solid medium containing 0.5% sucrose and 0.5% mannose. The plate was incubated at 30°C for 2 days. A single colony was isolated (strain MS-1). This strain was able to reach stationary phase overnight on sucrose plus a high concentration (up to 1%) of mannose. To stabilize the mutation, strain MS-1 was transferred daily to fresh Burk liquid medium with 0.5% sucrose plus 0.5% mannose for another 2 months. Thereafter, strain MS-1 was maintained in Burk agar with 0.5% sucrose plus 1% mannose. Revertants from strain MS-1 were isolated by growing strain MS-1 overnight on Burk medium with 0.5% sucrose plus 0.5% mannose. Cells were then serially diluted and plated onto Burk sucrose agar. Once grown, 2,500 colonies were randomly picked and transferred to Burk sucrose agar plates. Plates were incubated overnight at 30°C. Plates were then replica plated to Burk medium agar plates containing 0.5% sucrose and 0.5% mannose. Colonies that grew on sucrose but did not grow on sucrose plus mannose were isolated. The growth rates of these revertants in nutrient broth (Difco Laboratories, Detroit, Mich.) were compared with the growth rates of the wild-type cells as described previously (17).

Other assays. An oxygen uptake experiment was performed in a 10-ml oxygen chamber (model 5358; Yellow Springs Instrument Co., Yellow Springs, Ohio) at 30°C with an oxygen probe. Whole-cell N_2 fixation by *A. vinelandii* was done as described previously by Bishop et al. (2) Conditions for mixotrophic growth with H_2 and mannose were described previously (19). Protein was determined by the method of Bio-Rad Laboratories (Richmond, Calif.) with bovine serum albumin as standard.

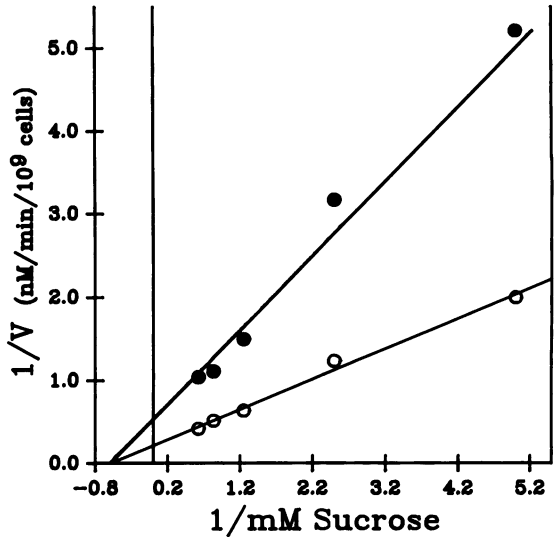

FIG. 1. Sucrose uptake in the presence (●) or absence (○) of 50 mM mannose. Double-reciprocal plot of uptake rate versus sucrose concentration by first-degree linear regression.

RESULTS

Sucrose uptake by *A. vinelandii* with or without mannose followed Michaelis-Menten kinetics. Figure 1 shows the double-reciprocal plot of substrate concentrations versus uptake rates. Results showed that the values of the apparent K_m of sucrose under both conditions were essentially the same (1.9 and 1.7 mM, respectively). However, the V_{max} values were significantly different. Without mannose, the V_{max} of sucrose was about 4.5 nmol/min per 10^9 cells. With 50 mM mannose, the V_{max} value decreased by almost one-half to 2.5 nmol/min per 10^9 cells.

The respiration rate of *A. vinelandii* grown on sucrose was monitored in the presence and absence of mannose (Fig. 2). Results showed that the rate of sucrose respiration was about 18 mM O_2 per min per 10^9 cells without mannose. Immediately after the addition of mannose, the respiratory rate of *A. vinelandii* decreased by 30% (12.5 mM O_2 per min

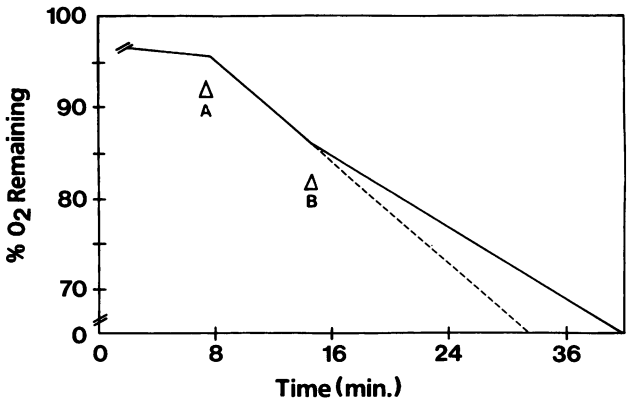

FIG. 2. O_2 uptake of *A. vinelandii*. A total of 8.1×10^7 cells was injected into the chamber containing 10 ml of air-saturated Burk medium at 30°C. A, Addition of sucrose (50 mM final concentration); B, addition of mannose (50 mM final concentration). ----, O_2 uptake rate without mannose addition.

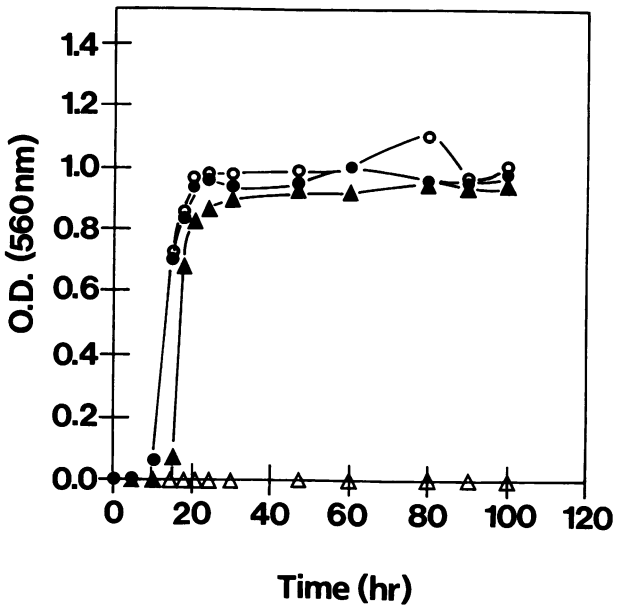

FIG. 3. Growth curves of *A. vinelandii* wild-type strain (○, △) and strain MS-1 (●, ▲) in Burk medium containing 0.5% sucrose (○, ●) or 0.5% sucrose plus 0.5% mannose (△, ▲). O.D. (560 nm), Optical density at 560 nm.

per 10^9 cells). The degree of respiration inhibition by mannose was independent of the O_2 concentration in the chamber.

In a similar experiment, the activity of nitrogenase (measured as acetylene reduction) was determined before and after mannose addition. The rate of acetylene reduction was inhibited after mannose addition. However, the degree of inhibition of acetylene reduction was also a function of O_2 concentration in the air phase during the assay. At low O_2 concentrations (less than 3% O_2), the rate of acetylene reduction was 512 nmol of C_2H_4 produced per h per 10^8 cells. Three minutes after mannose addition, the rate decreased to 356 nmol of C_2H_4 produced per h per 10^8 cells (or 70% activity remaining). When O_2 in the reaction chamber was close to 20%, the rate of acetylene reduction by cells growing on sucrose was about 460 nmol/h per 10^8 cells. Three minutes after mannose addition, the reduction rate was about 80 nmol/h per 10^8 cells (or 17% activity remaining).

We analyzed the mannose kinase, phosphomannose isomerase, and phosphoglucose isomerase in the cytoplasmic fraction of the wild-type cells. Results indicated that mannose was phosphorylated to mannose 6-P. It was subsequently converted to fructose 6-P and to glucose 6-P by their respective isomerases in the cytoplasm. The specific activities of mannose kinase, phosphomannose isomerase, and phosphoglucose isomerase were 110 (standard deviation, ±20) nM/min per mg of protein, 2 (standard deviation, ±1.4) nM/min per mg of protein, and 2,800 (standard deviation, ±263) nM/min per mg of protein, respectively.

A spontaneous mannose-resistant mutant (strain MS-1) was isolated. Strain MS-1 grew normally in 0.5% sucrose medium (Fig. 3). It grew almost as fast on sucrose medium with added mannose (0.5%). Figure 4 shows a double-reciprocal plot of the mannose uptake rate by the mutant and wild-type cells at various substrate concentrations. Results show that the apparent K_m and V_{max} for mannose of the

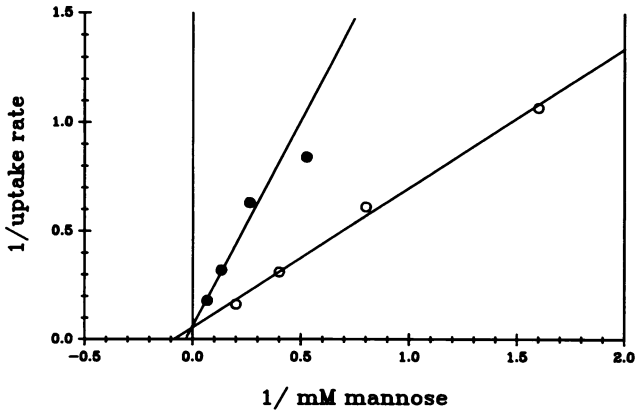

FIG. 4. Mannose uptake by *A. vinelandii* wild type (○) and by mutant MS-1 strain (●). Double-reciprocal plot of the uptake rate versus mannose concentrations by first-degree linear regression.

wild-type cells were 10 and 20 mM, respectively. In contrast, strain MS-1 had a much higher K_m (38 mM) for mannose uptake. The apparent V_{max} for mannose uptake by this mutant was about the same as that for the wild-type cell (17 nmol/min per 10^9 cells).

The effect of mannose on sucrose uptake by strain MS-1 was tested. Mannose did not inhibit the transport of sucrose in strain MS-1 (Fig. 5). Additionally, mannose did not decrease the respiration of strain MS-1 in sucrose medium. Strain MS-1 also failed to grow mixotrophically with H_2 and mannose. The enzymatic activities of mannose kinase, phosphomannose isomerase, and phosphoglucose isomerase in the cytoplasmic fraction of strain MS-1 were essentially the same as those of the wild-type cells.

We were able to isolate revertants from MS-1 at high frequency. Of 2,500 colonies picked, 3 colonies failed to grow on medium containing mannose. These revertants grew

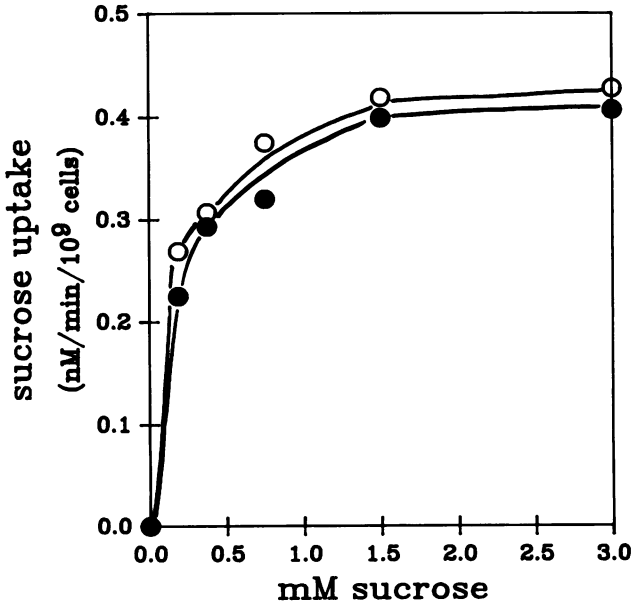

FIG. 5. Sucrose uptake by *A. vinelandii* MS-1 in presence (○) or absence (●) of 50 mM mannose at various substrate concentrations. Uptake rate represents nanomolar sucrose per minute per 10^9 cells.

normally in Burk medium under N_2-fixing conditions. Additionally, they have a generation time of 2.4 to 3.2 h when growing on complex medium such as nutrient broth. These rates were the same as those from the wild-type cells.

DISCUSSION

Uptake of a sugar can be inhibited by another sugar via several mechanisms (for a review, see reference 3). The uptake of sugars is influenced by the intracellular sugar phosphates (6), the electrochemical gradient (13), and a mechanism termed inducer expulsion (8). One of the better understood examples is the effect of glucose on the uptake of other substrates in *Escherichia coli* and *Salmonella typhimurium*. In these bacteria, many substrates are transported across the cell membrane by the phosphoenolpyruvate-dependent sugar phosphotransferase system (PTS). In the presence of glucose, the use of other sugars is blocked by components of the PTS system. For example, glucose inhibits the uptake of mannose by competing for the intracellular pool of the P-HPr protein inside the cell. Additionally, in the presence of glucose and other PTS sugars, enzyme III^{Glc}, a component of the PTS, is dephosphorylated. This dephosphorylated enzyme binds to many non-PTS substrate permeases and subsequently inhibits the transport of several sugars such as melibiose, maltose, lactose, and glycerol.

In this report, we showed that mannose inhibited sucrose transport noncompetitively. In vitro analysis showed that mannose could be phosphorylated to mannose 6-P by a kinase. Once formed, mannose 6-P could be isomerized to fructose 6-P and glucose 6-P by the cytoplasmic phosphomannose isomerase and phosphoglucose isomerase, respectively. However, the specific activity of phosphomannose isomerase was very low. Pindar and Bucke (12) examined the biosynthesis of alginic acid by *A. vinelandii*. They reported that the specific activities of phosphoglucose isomerase and phosphomannose isomerase in sucrose-grown cells were 3,000 and 0.2 to 2 nmol/min per mg of cytoplasmic protein, respectively. These values are in agreement with our results.

It is possible that a good portion of ATP was consumed to phosphorylated mannose by the kinase once this molecule was transported into the cell. However, the conversion of mannose 6-P to fructose 6-P was very slow. We hypothesize that the slow isomerization of mannose phosphate to fructose phosphate might effectively prevent the cell from using mannose. The accumulation of mannose and its phosphate derivatives inside the cell might also interfere with the sucrose permease. As a result, the uptake of sucrose also decreased.

The interaction of mannose resulting in growth inhibition is complex. A rather high concentration of mannose was needed (50 mM) to decrease the V_{max} of sucrose by about one-half. Yet, an earlier study (18) shows that at early lag phase, as little as 0.5 mM can delay cell division for more than 15 h. In that earlier study, we also showed that the degree of inhibition was influenced by the aeration rate. This suggested that the inhibitory effect of mannose on growth may not be totally due to the reduction of sucrose uptake.

Fixing N_2 aerobically requires extremely high energy input. The decrease in sucrose uptake after mannose addition coincided with a decrease in both respiration (Fig. 2) and acetylene reduction activity. The decrease in respiration and the decrease in acetylene reduction were likely two independent events, because the inhibition of respiration by mannose was not related to the O_2 concentration in the solution while the inhibition of acetylene reduction by mannose was highly influenced by the O_2 environment. Therefore, one likely possibility was that mannose caused a sudden decrease in electron supply by consuming ATP and by decreasing sucrose transport. As a result, the N_2-fixing cells became highly sensitive to their O_2 environment. This view is in agreement with the recent study by Kuhla and Oelze (7), which shows that the nitrogenase activity in response to oxygen stress of the enzyme is highly influenced by the rate of electron and energy supply to the nitrogenase.

A mutant (MS-1) resistant to mannose inhibition of sucrose-supported growth was isolated. Without mannose as a selective pressure, this mutant reverted to wild type at high frequency. Once reverted, cells grew normally. This suggested that mutation in strain MS-1 was quite specific. Strain MS-1 has a very high K_m for mannose transport. Since mannose could not be transported into these cells effectively, it would not consume ATP and would not generate phosphate intermediates to interfere with normal cell functions.

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