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Kinetics of Phosphate Uptake, Growth, and Accumulation of Cyclic Diphosphoglycerate in a Phosphate-Limited Continuous Culture of *Methanobacterium thermoautotrophicum*

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The archaebacterium *Methanobacterium thermoautotrophicum* was grown in continuous culture at 65°C in a phosphate-limited medium at specific growth rates from 0.06 to 0.28 h^{-1} (maximum growth rate [μ_{max}] = 0.36 h^{-1}). Cyclic-2,3-diphosphoglycerate (cyclic DPG) levels ranged from 2 to 20 mM in P_i-limited cells, compared with about 30 mM in batch-grown cells. The Monod constant for P_i-limited growth was 5 nM. P_i uptake rates were determined by following the disappearance of $^{32}P_i$ from the medium. Interrupting the H_2 supply stopped the uptake of P_i and the release of organic phosphates. Little or no efflux of P_i occurred in the presence or absence of H_2. P_i uptake of cells adapted to nanomolar P_i concentrations could be accounted for by the operation of one uptake system with an apparent K_m of about 25 nM and a V_{max} of 58 nmol of P_i per min per g (dry weight). Uptake curves at 30 μM P_i or above were biphasic due to a sevenfold decrease in V_{max} after an initial phase of rapid movement of P_i into the cell. Under these conditions the growth rate slowed to zero and the cyclic DPG pool expanded before growth resumed. Thus, three properties of *M. thermoautotrophicum* make it well adapted to live in a low-P environment: the presence of a low-K_m, high-V_{max} uptake system for P_i; the ability to accumulate cyclic DPG rapidly; and a growth strategy in which accumulation of P_i and cyclic DPG takes precedence over a shift-up in growth rate when excess P_i becomes available.

Transport studies in methanogenic bacteria are few, and no studies on P_i uptake have appeared (see reference 6 for a review). The usual approach to measurement of P_i uptake involves washing cells grown in batch culture with P_i-free medium. The cells are resuspended in the same medium, $^{32}P_i$ at an appropriate concentration is added, and samples of the suspension are collected at intervals onto membrane filters for counting. Such procedures may be unsuitable for *Methanobacterium thermoautotrophicum* because of the risk of harming the cells by exposure to traces of molecular oxygen and large temperature fluctuations. This methanogen derives energy for growth from the transfer of electrons from H_2 to CO_2 and has the intensive energy metabolism needed for a chemolithotrophic lifestyle at 65°C (27). ATP is synthesized during methanogenesis by a chemiosmotic mechanism (2, 18). Since interruption of H_2 supply causes exhaustion of the ATP pool within seconds, experimental conditions for transport studies have to be selected carefully.

Another problem with collecting cells and measuring their ^{32}P content is the rapid incorporation of ^{32}P into cellular components, particularly with an organism that accumulates cyclic-2,3-diphosphoglycerate (cyclic DPG). This phosphate metabolite is the main phosphorus compound in *M. thermoautotrophicum* (22). The unique characteristic of the cyclic DPG molecule is the diphosphate linkage between the hydroxyl groups at C-2 and C-3. This is the first reported biological occurrence of a pyrophosphate group in a seven-membered ring. Computer analysis indicated that the ring may prevent the pyrophosphate group of cyclic DPG from adopting the staggered conformation characteristic of other pyrophosphates (23).

The concentration of cyclic DPG was 40 mM in cells from H_2-limited chemostat cultures and 150 mM or higher in cells grown in batch cultures where H_2 mass transfer rates were inadequate for rapid growth (24). As shown in the present paper, the level of cyclic DPG was about 30 mM in cells from rapidly growing batch cultures and 2 to 23 mM in cells from P_i-limited chemostat cultures. In addition, a step-up in external P_i caused rapid accumulation of P_i in this metabolite. For this reason, P_i uptake measurements based on ^{32}P accumulated in the cells could be misleading.

The use of P_i-limited continuous cultures of *M. thermoautotrophicum* circumvented the above problems. In the steady state the extracellular P_i concentration was in the nanomolar range, and the kinetics of P_i uptake could be studied without stressing the cells. By following the rate of P_i disappearance from the medium after addition of $^{32}P_i$, we were able to demonstrate the presence of an uptake system for P_i which has an apparent K_m of 25 nM. Another interesting finding was that a step change in external P_i concentration resulted in a short-term uncoupling of uptake and growth and in a rapid expansion of the cyclic DPG pool. This finding suggests a growth strategy in which accumulation of P_i takes precedence over a shift-up in growth rate. A high capacity to sequester P_i from the environment shows that *M. thermoautotrophicum* is well adapted to live in low-P_i environments.

(Part of this study was presented in a lecture given by D.E.F. at the International Workshop on Biology and Biochemistry of Archaebacteria, Munich-Martinsried, June 27 to 30, 1985.)

MATERIALS AND METHODS

Growth of cells. *M. thermoautotrophicum* ATCC 29183 was cultured in a phosphate-limited medium at 65°C under an atmosphere of H_2 and CO_2 (4:1, vol/vol) at a pressure of

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200 kPa. A 1-liter chemostat vessel was used for 200-ml cultures (24). The medium was that of Schönheit et al. (20), modified as follows: Na_2HPO_4, either 10 or 50 μM, as indicated below; KCl, 2 mM; $NaHCO_3$, 9 mM; Na_2CO_3, 3 mM; NH_4HCO_3, 6 mM; NH_4Cl, 20 mM; nitrilotriacetic acid, 0.15 mM; $MgCl_2$, 0.2 mM; $FeCl_2$, 25 μM; $NiCl_2$, 5 μM; $CoCl_2$, 1 μM; and Na_2MoO_4, 1 μM. Resazurin was omitted. To improve buffer capacity, 50 ml of 0.4 M *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), titrated to pH 7.6 with aqueous NH_3, was added for each liter of final medium. The reducing agents, Na_2S and cysteine, were added last, after transfer of the medium to a 12-liter stainless steel reservoir. The medium was stored under H_2/CO_2 (4:1) at 200 kPa and was sterilized by membrane filtration en route to the culture vessels. The pH was monitored, but no pH control was necessary, because of the buffering capacity of the medium. The pH was constant at 7.0 (measured at 65°C).

Batch cultures were grown in the same medium, except that the concentrations of P_i and K^+ were changed to 2 and 12 mM, respectively. Otherwise, conditions were identical to those used for the chemostat cultures, with a culture volume of 200 ml in the chemostat vessel.

Exchange of headspace gases. The replacement of the H_2/CO_2 headspace gas with oxygen-free N_2 was accomplished by reducing the headspace pressure to 50 kPa and repressurizing to 200 kPa with N_2 15 times over a period of 1 min. The same procedure was used in reverse to restore the supply of H_2/CO_2 to the culture.

Sampling of cultures. Samples were collected via a sampling tube which extended into the culture and was fitted with a Teflon (E. I. du Pont de Nemours & Co., Inc., Wilmington, Del.) valve and a filter assembly (Acrodisc, 0.45 μm; Gelman Sciences, Inc., Ann Arbor, Mich.). Since the headspace gas was pressurized, removal and filtration of a 2-ml sample took about 2 s. The sampling line (internal volume, 0.4 ml) was flushed with about 1 ml of culture immediately before each sample was collected.

Extracellular P_i concentration. P_i concentrations were determined either by a colorimetric assay for P_i or by measuring $^{32}P_i$ radioactivity in the ambient medium. Two variations of the radiotracer method were used: a pulse method and a steady-state method.

In the pulse method, 1 ml of $^{32}P_i$ solution was injected into the culture to a final concentration of 10 μM $^{32}P_i$ (radioisotopic concentration, approximately 0.2 μCi/ml). Two or three 2-ml samples were withdrawn through the membrane filter at 2-min intervals. To distinguish between P_i and organic phosphates in the filtrate, P_i was converted to the antimony phosphomolybdate complex which was extracted from the solution (17). Antimony molybdate reagent (0.167 ml) and 1 ml of isoamyl alcohol were added to 0.833 ml of filtrate. After mixing for 10 s on a Vortex mixer, the aqueous and organic phases were separated by centrifugation at 15,600 × *g* for 30 s. The radioactivity of the extracted antimony phosphomolybdate complex was then counted via Cerenkov radiation.

Two 1-ml samples of the culture (cells and medium) were also taken for counting. Controls were performed to determine the efficiency of both the extraction procedure and the Cerenkov counting in isoamyl alcohol. Control experiments also showed that PP_i was quantitatively converted to P_i by the antimony molybdate reagent. Therefore, this method will overestimate the amount of $^{32}P_i$ in a filtrate if labeled PP_i and acid-labile organic phosphates are present.

The ambient P_i concentration in chemostat cultures was also determined by a steady-state approach in which the P_i in the inflowing medium was labeled with ^{32}P. After 10 or more generations, samples of the culture were filtered and analyzed for ^{32}P via extraction of the antimony phosphomolybdate complex as decribed above.

Phosphate transport measurements. Uptake experiments were run at methanogen concentrations from 0.02 to 0.10 mg (dry weight) of cells per ml and at P_i concentrations from 1 to 400 μM. Each kinetic run was initiated by rapid injection of 1 ml of $^{32}P_i$ solution into the chemostat culture. A stirring speed of 1,000 rpm, plus the turbulence created by inlet, outlet, and sampling tubes, ensured complete mixing within seconds. Two 1-ml samples of the culture (cells and medium) were removed immediately to determine the total amount of isotope added.

The frequency at which filtered samples were taken depended on the starting P_i concentration. With starting P_i concentrations above 1 μM, samples were collected at 1-min intervals or longer, using a new filter for each sample. A continuous sampling process was used when the starting P_i concentration was 1 μM. To resolve the time course of P_i uptake, 0.4- to 0.9-ml fractions were collected at 2- to 3-s intervals into tared scintillation vials. No volume effect on the Cerenkov count rate (counts per minute per milliliter) was observed for the range of volumes encountered by this sampling technique. Timing was marked at the beginning of each sample. It was necessary to change the filter after 10 to 12 fractions. The rate of P_i uptake was expressed as millimoles per minute per gram (dry weight). A_{660} was converted to dry weight of cells by using the previously established relationship grams (dry weight) per liter = 0.52 A_{660} (24).

Analysis of P_i uptake kinetics. Kinetic analysis was based on the premise that transport systems usually obey the Michaelis-Menten equation. To determine K_m, six to eight samples were collected when the P_i concentration was in the range of 2 to 0.2 K_m. For this reason, sample collection was started at 0.4 min in those experiments where the starting P_i concentration was 1 μM. If the timing was off by a few seconds, remaining P_i concentrations failed to bracket the K_m value. V_{max} and K_m were calculated by fitting each set of S,*t* values to the integrated form of the Michaelis-Menten equation: $V_{max}t = S_0 - S + K_m \ln (S_0/S)$, where S_0 = P_i concentration outside the cell after pulse addition of P_i to the culture, S = P_i concentration remaining at time *t*, V_{max} is the maximal uptake rate by a culture at saturation S, and K_m is the Michaelis constant (4, 5, 19).

Measurement of cyclic DPG. The spectrophotometric analysis for cyclic DPG was described previously (23). We have made an important change, however, in the extraction procedure. Unfiltered culture samples (20 ml) were chilled in an ice bath and centrifuged at 12,000 × *g* for 10 min. Cell pellets were frozen at −20°C for 12 h or longer. The pellet was thawed by adding 3 ml of 1 M HCl, and the cells were disrupted by sonication with a micro tip at 100 W for 5 s. The cell debris was removed by centrifugation at 18,000 × *g* for 15 min at 4°C. Internal concentrations of cyclic DPG were calculated by using the relationship for cell water of 1.8 ml/g (dry weight) of cells previously established by Schönheit and Perski (21).

Determination of total cellular phosphorus. Cells were grown in the presence of $^{32}P_i$ to constant specific radioactivity (six or more generations) in batch and chemostat cultures. Cells were collected on membrane filters and washed with 10 ml of reduced 0.1 M HEPES/Na^+, pH 7.0 (11), before determination of ^{32}P content.

TABLE 1. Total cellular phosphorus and cyclic DPG content of *M. thermoautotrophicum*

Growth conditions	Specific growth rate (h^{-1})	Total phosphorus (% cell dry weight)[a]	Cyclic DPG		Total P content minus P in cyclic DGP pool (% dry wt)
			μmol/g (dry wt)[a]	% of total P	
Batch	0.330–0.346	0.76 ± 0.05	50 ± 11[b]	40 ± 9	0.45 ± 0.08
P_i-limited continuous culture[c]	0.178	0.82 ± 0.06	42 ± 5	32 ± 4	0.56 ± 0.06
	0.170	0.66 ± 0.02	26 ± 3	24 ± 5	0.50 ± 0.03
	0.228	0.56 ± 0.04	4.0 ± 0.2	4 ± 1	0.54 ± 0.04
Mean					0.51 ± 0.05

[a] Mean ± standard deviation; three or four determinations.
[b] Mean ± standard deviation for 29 analyses from 8 cultures; no values omitted.
[c] Results from three chemostat runs. Samples taken after 10 or more generations into steady state.

RESULTS

Phosphorus and cyclic DPG content. *M. thermoautotrophicum* cells from P_i-limited continuous culture contained nearly as much phosphorus as cells from batch culture (Table 1). Cyclic DPG accounted for 4 to 40% of the total cellular phosphorus. The concentration of this cyclic pyrophosphate in P_i-limited cells was 2 to 23 mM, compared with about 30 mM in cells from optimized batch cultures, provided that internal cell water was 1.8 ml/g (dry weight) in each case. The reason for the large variation in the cyclic DPG content of cells from various chemostat runs is unclear. If one subtracts cyclic DPG phosphorus from total cellular phosphorus (Table 1), one notes that the phosphorus content of chemostat-grown cells shows no significant variation and is close to that of batch-grown cells.

We initially reported that cyclic DPG was below detection limits in cells from P_i-limited chemostat cultures (24). This discrepancy is due, in part, to the fact that we reported results obtained by direct HCl extraction of the cells without first disrupting them by freezing and sonication. Apart from this technical consideration, however, we have recently noted levels of cyclic DPG near 0.1 mM in two long-term chemostat runs lasting more than 120 generations. Thus, we cannot rule out the possibility that extended culture under P_i limitation resulted in the selection of variants of *M. thermoautotrophicum* in which the levels of cyclic DPG are maintained at low levels.

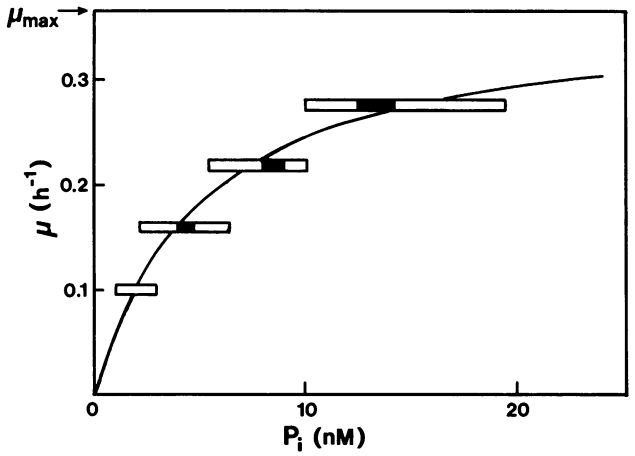

FIG. 1. Growth kinetics of *M. thermoautotrophicum* under P_i-limiting conditions. Specific growth rate versus ambient P_i concentration for a series of chemostat runs conducted over a period of a year. Individual chemostat cultures were maintained for 2 to 4 weeks. ▭, Range of P_i concentrations as determined by the pulse method. ▬, Range of P_i concentrations as measured by the steady-state method for two chemostat cultures in which the dilution rate was changed to higher values at 3-day intervals. Curve shows specific growth rate predicted by Monod equation with μ_{max} = 0.36 h^{-1} and K_μ = 5 nM.

Growth kinetics of P_i-limited continuous cultures. Specific growth rate versus P_i concentration for a number of chemostat runs is shown in Fig. 1. The concentration of P_i in the culture was the same at inflowing P_i concentrations of 10 and 50 μM, indicating that P_i was the limiting substrate. The μ_{max} of *M. thermoautotrophicum* growing at 65°C in optimized batch cultures was 0.36 h^{-1} (data not shown); this is the same value obtained earlier in this laboratory by using a chemostat-based technique (24). The value for the Monod constant, K_μ, which best fits the data in Fig. 1 was 5 nM.

It should be noted that our earlier estimate for K_μ of 4 μM was off by a factor of 1,000 (24). This overestimate was due to an error in the colorimetric assay for P_i caused by the redox indicator, resazurin, in the medium. For this reason, resazurin was omitted from the medium in the present work. Since P_i concentrations were in the nanomolar range, however, we had to use ^{32}P to measure the concentration of P_i in continuous cultures of *M. thermoautotrophicum*. As described above, we used two tracer methods to estimate the concentration of P_i. Both approaches gave similar results, so we feel that the data shown in Fig. 1 are reliable.

Response of growth rate to P_i pulse. Generally, an increase in the external concentration of the limiting nutrient brings about an increase in the growth rate. But in the case of *M. thermoautotrophicum* no growth, as measured by A_{660}, occurred for some time after a large pulse addition of P_i to a P_i-limited culture (Fig. 2). Although there was scatter in the duration of the lag period, the lag period shown in Fig. 2 was typical for a step change from 0.006 to 100 μM. Jumps to P_i concentrations between 200 and 400 μM resulted in lag periods up to 3 h (data not shown).

The external P_i concentration declined at a constant rate until growth resumed (Fig. 2). The downward break in the P_i uptake curve at the time growth resumed is probably due to the increase in cell density. Since a considerable amount of P_i disappeared from the medium before cell growth resumed, it is clear that a large increase in the phosphorus content of the cells was occurring. Thus, accumulation of this essential nutrient took precedence over a shift-up in growth rate.

P_i uptake is unidirectional. The time course for reappearance of radioactivity in the medium after the addition of 10 μM $^{32}P_i$ is shown in Fig. 3. Based on ^{32}P extracted as the antimony phosphomolybdate complex, the concentration of P_i in the medium dropped to 4 nM in about 4 min, then increased gradually, reaching values between 12 and 17 nM after 100 min. The appearance of labeled organic phos-

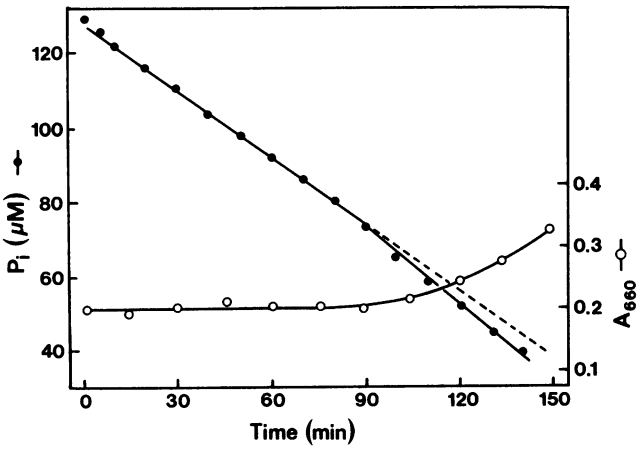

FIG. 2. Response of P_i-limited culture to a step change in P_i. At zero time, the inflow of medium into a chemostat operating at dilution rate of 0.17 h^{-1} was stopped, and P_i was added, to a final concentration of 132 μM. The steady-state concentration before the addition was about 5 nM. The amount of P_i added at zero time was sufficient for growth to a cell density of 0.5. Symbols: ●, external P_i concentration; ○, cell density, in terms of A_{660}.

phates, represented by nonextractable counts in the medium, occurred on a larger scale. Thus, we cannot rule out the possibility that part of the increase in phosphomolybdate-extractable counts was due to hydrolysis of inorganic pyrophosphate and acid-labile organic phosphates during the extraction procedure. Despite this uncertainty, the main conclusion from this experiment is that *M. thermoautotrophicum* can transport P_i against a large concentration gradient and that the movement of P_i across the cell membrane is unidirectional.

H_2 requirement for efflux of organic phosphates. The appearance of ^{32}P-labeled organic phosphates in the medium raises the possibility that cell lysis was occurring in the chemostat cultures. Since nonenergized cells are unable to regulate internal osmotic pressure, one way to test for cell lysis was to deprive the methanogen of H_2. The dashed line in Fig. 3 shows that H_2 depletion caused an immediate shutdown in the release of ^{32}P-labeled organic phosphate into the medium. Interrupting the H_2 supply had no measurable effect on the efflux of P_i from the cells, as indicated by the open squares in Fig. 3. Thus it appears that little or no cell lysis was occurring under the conditions of this experiment. An investigation into the nature of the compounds being released into the medium was beyond the scope of this paper.

H_2 requirement for P_i transport. One question that arises in regard to monitoring P_i uptake by following the disappearance of $^{32}P_i$ from the medium is whether other processes besides P_i transport into the cells may be occurring. One control experiment bearing on this point is shown in Fig. 4. The exogenous energy supply was interrupted by replacing the headspace gas with N_2. Four min later, 10 μM $^{32}P_i$ was added to the culture. The rate of $^{32}P_i$ disappearance from the medium, based on extraction of the antimony phosphomolybdate complex, was 0.03 mmol/min per g (dry weight). Since this rate was only 0.06% of the expected rate of uptake by energized cells at saturating P_i concentrations (see below), any losses of $^{32}P_i$ from the medium due to effects such as nonspecific absorption to the cell surface or chemostat vessel were negligible.

P_i transport began immediately after H_2 supply to the cells was restored (Fig. 4). The time for the P_i concentration to go from 10 μM to 4 nM was about 5 min, as compared with about 4 min for control cultures (e.g., as in Fig. 3). Since the 5-min period included any delay in energization of the cells due to the time for H_2 diffusion from the gas phase to the cells, any effect of short-term H_2 depletion on the P_i trans-

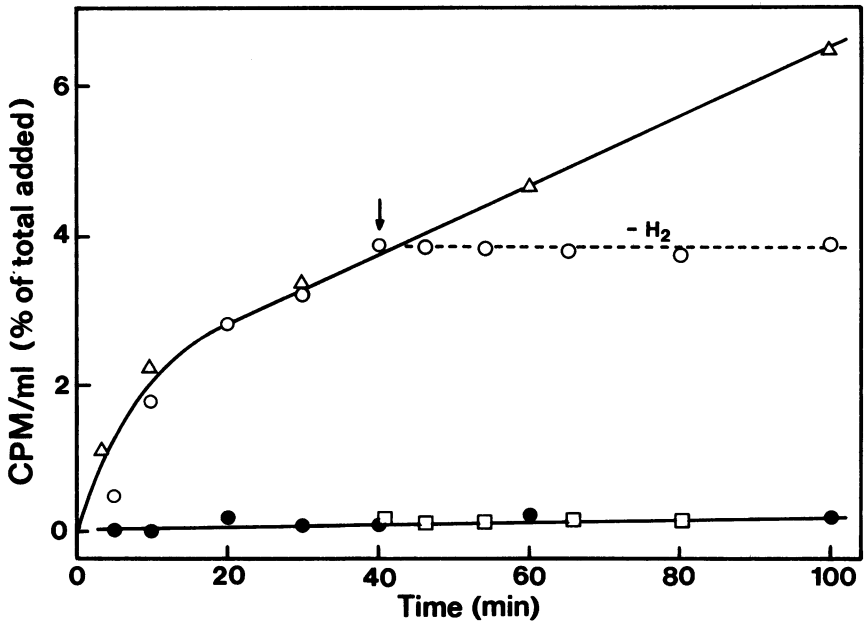

FIG. 3. Efflux of organic phosphate from *M. thermoautotrophicum* cells. At zero time, culture was switched from chemostat to batch mode, and $^{32}P_i$ was added, to a final concentration of 10 μM. Experiment 1: ●, radioactivity extracted from culture filtrate as antimony phosphomolybdate; △, nonextractable counts representing acid-stable organic phosphate. Experiment 2: H_2/CO_2 headspace gas was replaced with N_2 at time indicated by arrow; □, extractable counts; ○, nonextractable counts.

port system was minor. This experiment clearly establishes that P_i uptake by *M. thermoautotrophicum* is an energy-dependent process and that this organism does not maintain an endogenous energy source which can be used for this purpose.

Michaelis constant for P_i uptake system in cells adapted to nanomolar P_i concentrations. Radioactive P_i was injected into a P_i-limited chemostat culture at a final concentration of 1 μM. At a cell density of 0.02 mg (dry weight)/ml, the ambient P_i concentration returned to its steady-state value within 2 min. Within this short time, the efflux of radioactive P_i and phosphate metabolites from the cells was negligible. Thus, the disappearance of ^{32}P from the medium provided data for the evaluation of the Michaelis constant of the P_i uptake system.

A representative experiment is plotted in Fig. 5. The inset in Fig. 5 shows that data points from 1 to 0.1 μM P_i fall on a straight line. This is support for enzymelike transport kinetics, since the Michaelis-Menten equation becomes zero order with respect to substrate at substrate concentrations much greater than K_m.

Data points from the region where the P_i concentrations bracketed the apparent K_m are plotted on a larger scale in Fig. 5. The parameters V_{max} and K_m were calculated by fitting the data to the integrated form of the Michaelis-Menten equation by nonlinear regression analysis (19). For the experiment shown in Fig. 5, the values $K_m = 25 \pm 8$ nM and $V_{max} = 58 \pm 2$ mmol/min per g (dry weight) were obtained. The lines in Fig. 5 show the predicted time course for P_i uptake when these values, together with a final P_i concentration of 6 nM, are used for the calculation. Although technical difficulties, and perhaps variability in the cultures, prevented a more accurate determination of K_m, the results show that cells that are adapted to nanomolar P_i concentrations have a high-affinity P_i uptake system.

Capacity of transport system relative to rate of P_i assimilation. At steady state, the rate of P_i uptake is equal to the rate of P_i assimilation. In the experiment shown in Fig. 5, the rate of P_i uptake by the chemostat culture was 0.28 mmol/min per g (dry weight) before the step change in external P_i. After the step change, the uptake rate increased to a V_{max} of 58 mmol/min per g (dry weight). This 200-fold rate increase demonstrates the high capacity of the P_i uptake system in cells adapted to low P_i concentrations.

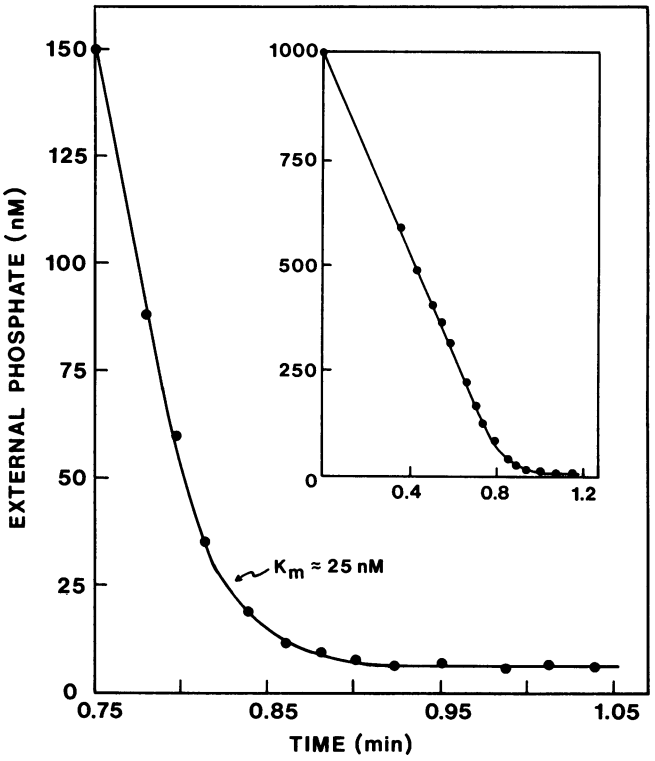

FIG. 5. Kinetics of P_i uptake. Chemostat culture: 65°C; dilution rate, 0.173 h^{-1}; rate of P_i input, 2.88 nmol/min. At zero time, 199 nmol of $^{32}P_i$ was added, changing the external P_i concentration from approximately 5 to 1,000 nM. Results from a single experiment are plotted on two scales. Curve shows time course of P_i uptake predicted by integrated Michaelis-Menten equation with $K_m = 25$ nM and $V_{max} = 58$ mmol/min per g (dry weight) of cells; a small correction for inflowing P_i was made.

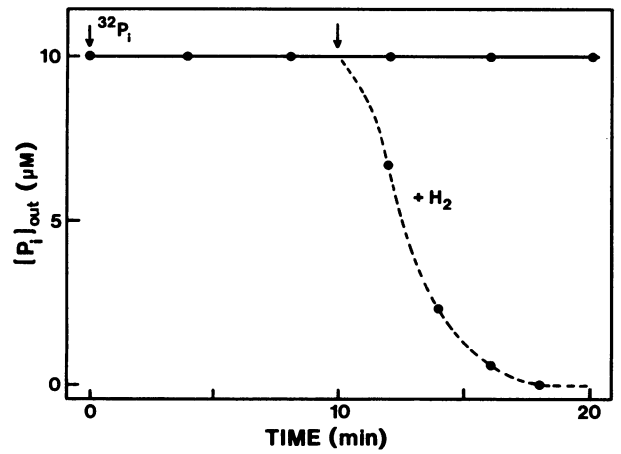

FIG. 4. H_2 requirement for P_i transport. At −5 min, H_2/CO_2 atmosphere of chemostat operating at a dilution rate of 0.17 h^{-1} was replaced with N_2. At zero time, culture was switched to batch mode, and $^{32}P_i$ was added at 10 μM. In a parallel experiment, original H_2/CO_2 atmosphere was restored at time indicated by arrow at right.

Reduction in V_{max} at high P_i concentrations. A number of uptake experiments were performed at higher P_i concentrations to test for the presence of a second P_i uptake system with a higher K_m. Although P_i concentrations as high as 0.4 mM were tested, no evidence for a transport system having a K_m near 1 mM was obtained.

These experiments showed, however, that the maximal rate of P_i uptake was reduced at high P_i concentrations (Fig. 6). The curve labeled AB shows the time course for P_i disappearance after a 30 μM pulse. The rate of P_i uptake slowed to 8.2 mmol/min per g (dry weight) in about 8 min, then remained constant along the B curve until most of the P_i had disappeared. The zero-order rate for the slow phase (designated V'_{max} to distinguish it from V_{max}) was 8.1 ± 0.6 mmol/min per g (dry weight) for seven experiments at extracellular concentrations ranging from 30 to 400 μM (data not shown). Thus, V'_{max} was remarkably independent of the size of the P_i impulse.

It is convenient to define the initial phase of rapid uptake by extrapolation of the zero-order curve B back to zero time and to use the drop C in external P_i concentration as a measure of the initial phase. Thus, the initial phase for the experiment shown by curve AB in Fig. 6 corresponded to a drop in the external P_i concentration of 6 μM. Similar C

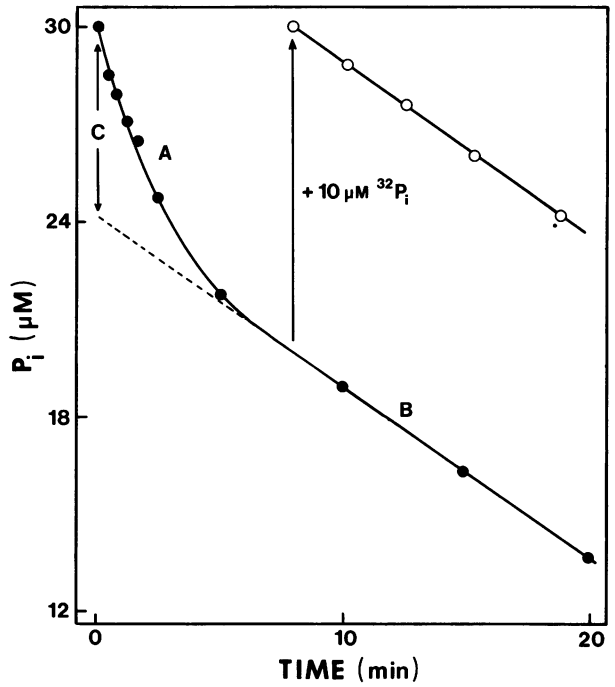

FIG. 6. Time course for P_i uptake after culture was switched from chemostat to batch mode and P_i was added to a final concentration of 30 μM (line AB). In a parallel experiment (○), the external P_i concentration was stepped up another 10 μM at 8 min by addition of $^{32}P_i$. C, amount of P_i taken up during initial phase (A) of transport.

values were observed at starting P_i concentrations of 30 to 200 μM (data not shown). In contrast, when the external P_i concentration was 1 μM, no biphasic behavior was observed (Fig. 5). The V_{max} obtained when the starting concentration at 1 μM was 58 mmol/min per g (dry weight). This sevenfold difference between V_{max} and V'_{max} suggests that the transport system is subject to some type of feedback inhibition resulting from the rapid movement of P_i into the cell.

To test whether the change from V_{max} to V'_{max} is caused by rapid filling of the intracellular P_i pool, a second pulse of P_i was added to the culture at 8 min (Fig. 6). Radioactive P_i was used for the second pulse, and the rate of P_i removal from the medium was monitored by extracting the antimony phosphomolybdate complex. There was no initial phase of rapid transport and, as expected, the rate of P_i uptake was the same as the ongoing rate at the time of second impulse. Thus accumulation of P_i, or some other time-dependent process associated with rapid influx of P_i, was responsible for the decrease in V_{max} that occurred with large pulses of P_i. If one assumes that no significant amount of P_i leaves the intracellular P_i pool during the initial period of rapid P_i accumulation, one finds that a 6 μM decrease in external P_i corresponds to an increase in the intracellular pool of about 31 mM. Thus one may reasonably attribute the observed decrease in V_{max} to accumulation of intracellular P_i in amounts high enough to interfere with transport.

Response of cyclic DPG pool to P_i impulse. The time course for accumulation of cyclic DPG following a jump from 0.006 to 30 μM P_i indicated a steady influx of P_i into the cyclic DPG pool (data not shown). The pool expanded by 50% in 18 min, by which time one-third of the added P_i had been transported into the cells. Since only 20% of the incoming P_i was accumulating in the cyclic DPG pool, it will be necessary to look at other phosphorus components in the cell to account fully for the change in phosphorus content occurring before the step-up in growth rate.

DISCUSSION

The major effect of P_i limitation was on the cyclic DPG pool (Table 1). However, the supply of H_2 also appears to be an important factor in determining the level of this cyclic pyrophosphate. We have previously reported that cyclic DPG levels were high in cells grown under conditions of H_2 restriction, either in batch or continuous culture (24). As shown here, cyclic DPG levels were reduced when H_2 was abundant, leading to a reduction in total phosphorus in batch-grown cells from 1.7 to 0.8%. Although the level of cyclic DPG varied from one P_i-limited chemostat run to another, steady-state values for total phosphorus and cyclic DPG content were frequently identical to those of fast-growing batch cells (Table 1). As discussed below, K^+ levels in the medium also have an effect on the concentration of this metabolite, so we are far from a complete understanding of the factors involved in regulation of cyclic DPG levels in *M. thermoautotrophicum*.

Since growth in P_i-limited situations often produces striking changes in microorganisms, it is of interest to contrast these effects with the limited impact of P_i restriction on *M. thermoautotrophicum*. In gram-positive bacteria, the cell wall teichoic acids, which can contain more than 30% of the total phosphorus of exponential-phase cells, are replaced with teichuronic acid, an anionic polymer which contains no phosphate (8, 9). Another effect of P_i limitation is the partial replacement of phospholipids by phosphorus-free lipids (1, 14, 16). Thus, growth under conditions of restricted phosphorus supply results in a reduction in phosphorus content, whereas P_i-limited *M. thermoautotrophicum* cells had a total phosphorus content comparable to that of cells from optimized batch cultures.

We attribute this to the frugal phosphorus economy of the methanogen. The rod-shaped gram-positive members of the genus *Methanobacterium* exhibit a distinct cell wall which contains pseudomurein but no phosphorus (12). In addition, the glycerol diethers and tetraethers of the membrane lipids of thermophilic methanogens are mixtures of glycolipids and phosphoglycolipids (13, 26). The main polar head groups include two different disaccharides, glycerolphosphate, or a free hydroxyl. These differences—the lack of teichoic acids and the fact that disaccharides or a free hydroxyl are used as polar head groups in the membrane lipids—suggest that *M. thermoautotrophicum* has evolved with a minimal phosphorus content in the structural components of the cell membrane and cell wall. Therefore, changes in the cell's phosphorus content under conditions where the supply of P_i is restricted are not likely to involve changes in structural components. Perhaps this is why changes are seen mainly in the cyclic DPG pool.

The next point of interest is the P_i uptake system of *M. thermoautotrophicum*. Because of a Monod constant of 5 nM, the steady-state concentration of P_i was below 10 nM in P_i-limited chemostat cultures. By using the integrated form of the Michaelis-Menten equation, we were able to take advantage of this fact to monitor P_i transport across the cell membrane of this methanogen without perturbing the cells in any way. The integrated form of the Michaelis-Menten equation defines the time course of P_i disappearance from the medium and allows one, in principle, to obtain accurate values of the kinetic parameters from a single experiment.

The uptake process can be monitored even if there is significant flow of P_i from the intracellular P_i pool into other cellular pools. The main finding is that *M. thermoautotrophicum* has an avid facility for sequestering P_i as reflected by the presence of a low-K_m, high-V_{max} uptake system and the ability to store incoming P_i as cyclic DPG.

In many microorganisms, P_i uptake depends on the operation of two uptake systems, one with a high K_m for P_i and one with a low K_m. Rhizobia, however, have a single P_i uptake system which is repressible over a 10- to 180-fold range in activity in different rhizobial species (25). The rhizobial uptake system displays K_m values ranging from 1.6 to 6 μM—typical values for low-K_m P_i uptake systems in eubacteria (see reference 5 for other examples and references). Smaller values have been reported for various cyanobacteria, algae, and dinoflagellates; for example, K_m values of 15 and 500 nM have been published for the cyanobacterium *Synechococcus* (10, 15), whereas values ranging from 5 to 16 nM have been reported for several dinoflagellates (7). For comparison, the K_m for the methanogen system observed in this paper was about 25 nM.

The results presented here suggest that only one transport system was operative in *M. thermoautotrophicum* cells adapted to nanomolar P_i concentrations. Our argument against the presence of a constitutive high-K_m system is based on the kinetics of P_i uptake at concentrations between 30 and 400 μM. In this range, the uptake rate during the slow phase was independent of the external P_i concentration. This result is difficult to reconcile with the presence of a second transport system having a high K_m and a maximum velocity V'_{max}. For example, with K_m = 1 mM, the predicted uptake rates at 30 and 400 μM P_i would be 0.03 and 0.29 V'_{max}, respectively. Since the observed uptake rate was constant at $V'_{max} = 0.14\ V_{max}$, we believe that V'_{max} is associated with the inhibited low-K_m system (see below) and that no high-K_m system was operative in *M. thermoautotrophicum* cells adapted to P_i-limiting conditions.

It should be noted that no P_i uptake measurements using cells grown in media containing high concentrations of P_i are available yet, so one cannot rule out the presence of a high-K_m system under those conditions. It will be important in the future to investigate the transport of P_i by cells growing in media containing millimolar concentrations of P_i.

Since the presence of a constitutive high-K_m system seems unlikely, the next issue concerns the decrease from V_{max} to V'_{max} accompanying a large increase in total cellular phosphate. This effect suggests the possibility of feedback inhibition by P_i. While P_i-limited cells doubtless have the ability to fill the intracellular P_i pool when excess P_i is available, other phosphate metabolites may be acting as regulatory signals. For example, expansion of the cyclic DPG pool was rapid enough to act as a feedback signal to limit the uptake rate of P_i. Further studies will be directed at measuring the levels of P_i in the cells to establish if P_i plays a role in the regulation of the activity of the low-K_m P_i uptake system.

Another unresolved issue concerns the source of energy to drive P_i accumulation against large concentration gradients. We have shown that interrupting the H_2 supply abolished P_i uptake. This finding demonstrates the need for metabolic energy but no evidence that uptake is coupled to ATP hydrolysis is available. Metabolic energy was also required for the release of organic phosphate from the cells.

In a P_i-limited chemostat the cells must take up P_i at a rate sufficient to meet biosynthetic demands. Our results show that *M. thermoautotrophicum* has the capacity to transport P_i into the cells at rates markedly greatly than the minimal rate needed for obligatory biosynthetic requirements. In fact, a step change from 6 nM to 1 μM P_i resulted in a 200-fold increase in the uptake rate. Larger step changes caused a transitory uncoupling of uptake and growth. This slowing of growth rate was in contrast to the immediate adjustment by the cells to a new growth rate observed during a shift-up in dilution rate in H_2-limited chemostat cultures (24). In the experiments reported here, a large increase in both cyclic DPG and total cellular phosphorus preceded the step-up in growth rate. A similar phenomenon occurs when P_i-limited chemostat cultures of *Monochrysis lutheri* are pulsed with P_i (3). Growth of the algal cells was interrupted while accumulation of polyphosphate proceeded. The pulse resulted in synchronization of the cells when accelerated growth resumed.

K^+-limited cultures of *M. thermoautotrophicum* also respond to an up-shift in limiting nutrient by delaying the step-up in growth rate for one or more generation times (R. D. Krueger, R. J. Seely, and D. E. Fahrney, Syst. Appl. Microbiol., in press). During this lag period, the internal K^+ concentration increased from 320 to 800 mM, and cyclic DPG rose from a range of 20 to 30 to a range of 150 to 180 mM. The delay in accelerating the growth rate in response to a pulse of either K^+ or P_i suggests a growth strategy in which growth is uncoupled from P_i uptake to allow stockpiling of this essential nutrient.

In conclusion, *M. thermoautotrophicum* appears well adapted to sequester P_i from low-P_i environments. We believe that the P_i uptake system of this organism is especially interesting in terms of capacity. Although the systems of certain cyanobacteria and dinoflagellates may have comparable K_m values, V_{max} values are generally lower than the value for the methanogen system. Dinoflagellates and cyanobacteria adapted to low-P_i environments can be expected to exhibit Monod constants in the nanomolar range, as is the case for *M. thermoautotrophicum*. But the methanogen must have a much larger V_{max} to scavenge P_i at the high rates needed for growth at 65°C at doubling times as fast as 2 h. Further flexibility in handling P_i efficiently requires a mechanism for storing P_i during periods when this nutrient is abundant. Evidently, this is possible in the methanogen because of cyclic DPG, whereas most microorganisms use polyphosphate. Finally, the energetic cost of P_i-limited growth is rather high, but this also appears to pose no problem for *M. thermoautotrophicum*.

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