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Changes in the cellular energy state affect the activity of the bacterial phosphotransferase system

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The effect of different cellular free-energy states on the uptake of methyl α -D-glucopyranoside, an analogue of glucose, by the *Escherichia coli* phosphoenolpyruvate:carbohydrate phosphotransferase system was investigated. The intracellular [ATP]/[ADP] ratio was varied by changing the expression of the *atp* operon, which codes for the H⁺-ATPase, or by adding an uncoupler of oxidative phosphorylation or an inhibitor of respiration. Corresponding initial phosphotransferase uptake rates were determined using an improved uptake assay that works with growing cells in steady state. The results show that the initial uptake rate was decreased under conditions of lowered intracellular [ATP]/[ADP] ratios, irrespective of which method was used to change the cellular energy state. When either the expression of the *atp* operon was changed or 2,4-dinitrophenol was added to wild-type cells, the relationship between initial phosphotransferase uptake rate and the logarithm of the [ATP]/[ADP] ratio was approximately linear. These results suggest that the cellular free-energy state, as reflected in the intracellular [ATP]/[ADP] ratio, plays an important role in regulating the activity of the phosphotransferase system.

Keywords: glucose phosphotransferase system; H⁺-ATPase; [ATP]/[ADP] ratio; phosphoenolpyruvate; metabolic inhibitors.

Escherichia coli can use any one of a large variety of compounds as the sole carbon source for growth. In the presence of a mixture of compounds, this bacterium will often show a preference for certain carbohydrates, such as glucose, and repress the expression of uptake systems for other compounds (inducer exclusion [1]). During growth on a different carbon source such as succinate, however, *E. coli* maintains a relatively high capacity for glucose transport [1]. The bacterial phosphoenolpyruvate:carbohydrate phosphotransferase system catalyses the uptake and concomitant phosphorylation of a variety of carbohydrates, for example glucose [1]. The phosphotransferase system is a group-transfer pathway consisting of two soluble phosphoproteins (Enzyme I and HPr) and, for each carbohydrate, a specific protein or protein complex, the carbohydrate permease (for *E. coli* in the case of glucose, soluble Enzyme IIA^{Glc} and membrane-bound Enzyme IICB^{Glc}). During the uptake of a glucose molecule, for example, a phosphoryl group from phosphoenol-

pyruvate is transferred sequentially along Enzyme I, HPr, Enzymes IIA^{Glc} and IICB^{Glc}, to the glucose molecule.

Metabolic activity and changes in the cellular energy state have been shown to affect phosphotransferase activity in a variety of ways: the addition of substrates to activate respiration reduced the steady-state accumulation of methyl α -D-glucopyranoside (MeGlc) [2, 3], a non-metabolisable analogue of the glucose phosphotransferase system; the inhibition of MeGlc uptake by respiration was more severe in *atp* mutants, which cannot use the protonmotive force to synthesise ATP [4]; the inhibition by oxidisable substrates could be reversed by the addition of uncouplers of oxidative phosphorylation such as carbonyl cyanide *m*-chlorophenylhydrazone [2] or 2,4-dinitrophenol [5] and the respiratory inhibitor sodium azide [2, 5]. Uncouplers even stimulated MeGlc uptake in the presence of an oxidisable substrate [6], and this stimulation was much greater under aerobic than under anaerobic conditions. Furthermore, the addition of substrates of the respiratory chain, such as D-lactate or ascorbate, caused inhibition of MeGlc uptake by membrane vesicles [6]. All these results appeared paradoxical as phosphotransferase system activity requires input of intracellular free energy in the form of phosphoenolpyruvate. Diametrically opposite results were also obtained: in the absence of oxidisable substrates, uncouplers inhibited the rate of MeGlc uptake into whole cells [3]. The incubation of *E. coli* cells with high arsenate and low phosphate, which lowered intracellular ATP and phosphoenolpyruvate levels, inhibited partially the steady-state uptake of MeGlc [7]. Preincubation with sodium azide and sodium fluoride (which inhibits enolase), led to complete inhibition of MeGlc uptake [8].

The effects of metabolic inhibitors also depended on the carbon source used for growth: in cells grown on glucose, both

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Abbreviations. Phosphotransferase system, phosphoenolpyruvate:carbohydrate phosphotransferase system; HPr, histidine-containing protein; Enzyme I, phosphoenolpyruvate–protein phosphotransferase; Enzyme II, protein-*N*-phosphohistidine–sugar phosphotransferase; IIA^{Glc} and IICB^{Glc}, Enzymes II of the phosphoenolpyruvate:glucose phosphotransferase system; MeGlc, methyl α -D-glucopyranoside; IPTG, isopropyl thio- β -D-galactoside.

Enzymes. Enzyme I of the phosphotransferase system, phosphoenolpyruvate–protein phosphotransferase (EC 2.7.3.9); Enzyme II of the phosphotransferase system, protein-*N*-phosphohistidine–sugar phosphotransferase (EC 2.7.1.69).

KCN and dinitrophenol stimulated the initial MeGlc uptake rate by themselves, but only KCN increased steady-state uptake levels as well [9, 10]. With lactate as carbon source, both initial rate and steady-state uptake were decreased by KCN, whereas dinitrophenol stimulated the initial uptake rate and did not affect steady-state accumulation [9, 10]. In few studies was the cellular free-energy state actually monitored and modulated in more than one way.

The above-mentioned effects of changes in the cellular energy state on the phosphotransferase system were not measured in steadily growing cells, but in harvested and resuspended cells. In addition, these studies did not determine initial uptake rates under steady-state conditions of the cellular energy state, nor did they measure the [ATP]/[ADP] ratio. Consequently, the interesting questions of whether phosphotransferase activity correlates with the cellular energy state in terms of the [ATP]/[ADP] ratio under steady-state growth conditions, and whether this correlation is independent of the method by which the [ATP]/[ADP] ratio is varied, remained unanswered. In this study, we investigate the effect of steady-state changes in the cellular energy state on the initial rate of phosphotransferase uptake. The intracellular [ATP]/[ADP] ratio was varied by changing the expression of the *atp* operon, or by adding the uncoupler dinitrophenol or the respiratory inhibitor potassium cyanide. We show that lowering the cellular energy state by all methods caused a concomitant decrease in the initial MeGlc uptake rate.

MATERIALS AND METHODS

Bacterial strains. The *E. coli* strain used as wild type in this study, PJ4004, has the genotype F^+ , *asnB32*, *thi-1*, *relA1*, *spoT1*, *lacUV5* and *lacY*(Am). In strain PJ4002, the *atp* promoters [11] have been replaced by the *tacI*p promoter. In strain PJ4000, the *atp* promoters have been replaced by the *lacUV5*p promoter. Strains PJ4004, PJ4002, and PJ4000 were derived from LM3118 [12], LM3113 and LM3112 [13], respectively, by transformation with the plasmid pBR322 [14].

Growth of bacterial cultures. The cells were grown at 30°C in minimal Mops medium [15], supplemented with 2.5 µg/ml thiamine, 100 µg/ml ampicillin, 18.5 mM succinate and isopropyl thio-β-D-galactoside (IPTG) to the required concentration. To ensure exponential growth of the cells at steady state for at least six generations before sampling, the cultures were inoculated from a pre-culture of known absorbance, using previously determined generation times to calculate the required dilution so that the absorbance at 600 nm (A_{600}) did not exceed 0.2 the next day. The pre-culture consisted of cells that were already growing exponentially; for PJ4002 and PJ4000, 10 µM and 100 µM IPTG, respectively, were included.

Measurement of cell density. To determine the absorbance, 1 ml volumes of growing cell cultures were fixed by mixing with 200 µl 37% formaldehyde [15]. The samples were stored at 4°C for up to 24 h before the A_{600} was measured with a spectrophotometer. Absorbance values were corrected for dilution by formaldehyde. For a culture of $A_{600} = 0.1$, 1 ml was equivalent to 31.5 µg dry cell mass.

Measurement of intracellular [ATP]/[ADP] ratio. Samples of 900 µl were withdrawn from the cultures and mixed immediately with an equal volume of hot phenol [80°C, equilibrated with Tris/EDTA (10 mM Tris, 1 mM EDTA, pH 8) and containing 0.1% 8-hydroxyquinoline] by vortexing vigorously for 10 s. The samples were left to stand at room temperature for at least 1 h, vortexed again, stored at -20°C for up to several weeks, thawed, and centrifuged at 13000 rpm for 2 min in an Eppendorf centrifuge. The phenol was extracted from the water

phase with an equal volume of chloroform. ATP and ADP were assayed at room temperature, using a luciferin-luciferase ATP monitoring kit (LKB) as recommended by the supplier. The assay was carried out in the presence of 3 mM phosphoenolpyruvate. ADP was assayed after ATP had been determined by adding pyruvate kinase and recording the increase in luminescence. The results were corrected for ADP contamination in the phosphoenolpyruvate preparation and for quenching of the signal by the addition of pyruvate kinase.

Transport of methyl α-D-glucopyranoside. For the initial studies (Table 1), transport assays were carried out as described previously [16]. To summarise, cells ($A_{600} \approx 0.5$) were harvested by centrifugation at 4°C, washed twice with minimal Mops medium excluding carbon source, and resuspended to 100 times the original density. The suspension was diluted 30:70 (by vol.) with Mops medium (without carbon source) and aerated for 3 min. The uptake assay was then initiated by adding [U-¹⁴C]MeGlc (Amersham, specific activity: 100 µCi/mmol) to the cell suspension to a final concentration of 500 µM. Samples of 100 µl were withdrawn at 10-s intervals, quenched in 10 ml Mops medium at room temperature, and filtered rapidly through glass-fibre filters (Whatman GF/F). The exact time of quenching was noted with a stop-watch. 500 µl demineralised water and 4 ml Packard Scintillator Plus scintillation cocktail were added to each filter in scintillation vials before measuring the radioactivity in a liquid scintillation counter.

In subsequent experiments, this assay was modified to work with steadily growing cells. 1.5 µmol [U-¹⁴C]MeGlc (specific activity: 300 µCi/mmol) was preincubated in the reaction vessel in a thermostatted water bath at 30°C. The assay was initiated by adding 3 ml growing cell culture (A_{600} 0.12–0.2) to the labelled sugar and vortexing briefly. Four samples (0.5 ml) were withdrawn at time intervals of approximately 4 s. The cells were quenched in 10 ml ice-cold growth medium. The exact time of quenching was noted with a stop-watch. The samples were filtered as described above; in addition, the filters were washed with 5 ml ice-cold growth medium. Further sample processing and counting was performed as described above. The background activity bound to the filters was determined by performing the procedure with 3 ml medium instead of cells.

RESULTS

To examine a possible relationship between free-energy metabolism and phosphotransferase system activity, we compared sugar uptake in three *E. coli* cultures that differed in expression of the H^+ -ATPase. Initially, we employed the standard transport assay [16] to measure phosphotransferase system activity, which determines uptake of the glucose analogue MeGlc into cells resuspended in medium after concentration through centrifugation. Table 1 shows the uptake rate of MeGlc of the wild-type (PJ4004) and the *atp*-inducible (PJ4002) strains. In the strain PJ4002, the chromosomal *atp* genes are cloned behind the *tacI*p promoter. Expression of the H^+ -ATPase in this strain varies between approximately 20% of the wild-type level in the absence of IPTG and 400% of the wild-type level when the operon is fully induced [13]. The steady-state generation times of the cultures agreed very well with published results [17]. Table 1 shows that there was little change in the initial uptake rate of MeGlc, even when the [H^+ -ATPase] was varied over a large range; when the cells contained 20% of the normal level of H^+ -ATPase (PJ4002 without IPTG [13]), the uptake rate was 22% lower than normal.

We then measured the intracellular [ATP]/[ADP] ratio in the cells that were used for the uptake experiment to verify their

Table 1. Steady-state growth rates of strains and initial MeGlc uptake rates obtained with the standard assay procedure. Cells were grown as described with the indicated IPTG concentrations in the growth medium. Transport assays were performed according to the standard procedure as described in the text. Initial uptake rates were normalised with respect to the $14 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ dry cell mass observed in the wild-type strain PJ4004. Data reflect the initial uptake rate $\pm$ standard deviation. The number of determinations is shown in parentheses.

Strain	[IPTG]	Generation time	Relative MeGlc uptake
	μM	min	
PJ4004	0	125	1.00 ± 0.04 (2)
PJ4002	0	230	0.78 ± 0.07 (2)
PJ4002	20	160	1.02 ± 0.08 (4)

Fig. 1. The [ATP]/[ADP] ratio during aeration of a dense cell suspension of strain PJ4004. Aeration was performed as in the procedure for the standard uptake assay. Samples for ATP and ADP analysis were taken at the indicated time intervals. The [ATP]/[ADP] ratio in the suspension was 2.3 before aeration commenced. In the standard uptake assay, labelled carbohydrate was added 3 min after commencing with aeration and samples were withdrawn at 10-s intervals.

steady energy state (Fig. 1). The [ATP]/[ADP] ratio in the cells was very low (2.3), presumably because the cells had been centrifuged and washed in the absence of carbon source, and had become anaerobic. When the cells were mixed and aerated, the [ATP]/[ADP] ratio increased to 3.3 (time zero in Fig. 1), but decreased steadily during the time of aeration. When labelled carbohydrate was added to the cells during a standard uptake assay (at time 3 min) they were clearly not at a steady energy state. More importantly, the [ATP]/[ADP] ratios measured here were very much lower than the normal steady-state ratio of 11 during exponential growth on succinate.

To measure MeGlc uptake under cellular free-energy states that reflected the state at which the cells were growing, the standard uptake assay procedure was adapted to work with steadily growing cells. Because the cells could no longer be concentrated and had to be kept at low cell density to remain at steady state [13], this entailed increasing the specific activity of MeGlc in the assay by a factor of three, using larger sample volumes, including growth substrate under transport assay conditions, and thermostating the assay at 30°C . Under these conditions the [ATP]/[ADP] ratio did not change significantly during the time course of an uptake experiment: four samples taken within 30 s of MeGlc addition to the assay had an average [ATP]/[ADP] ratio of 10.3 with a standard deviation of 1.2. This value is within the experimental error of the ATP and ADP determination (Tables 2 and 3). The measured [ATP]/[ADP] ratios also corresponded to the normal steady-state values [17].

Table 2 summarises the uptake data for bacterial cultures that were grown under exactly the same conditions as in the experiment in Table 1, except that the modified uptake assay procedure was used; the steady-state [ATP]/[ADP] ratios during growth for the three cultures are also presented. Three observations are important: First, as expected [17], the [ATP]/[ADP] ratio varied over a large range from 4.3 to 12 in the strain PJ4002 when amounts of 0 μM and 20 μM IPTG, respectively, were added. The wild-type strain had a ratio of 11. Secondly, the different cellular energy states were correlated with different initial MeGlc uptake rates. The strain PJ4002 had 79% of the wild-type uptake rate, whereas the uptake rate increased to 110% of the wild-type level when 20 μM IPTG was included in the growth medium. For the strain PJ4002 grown without IPTG, the decrease of 21% in uptake rate with respect to the wild type was approximately the same as for the standard assay method; nevertheless, the modified method is preferred because the [ATP]/[ADP] ratios corresponded to the steady-state values. Thirdly, the absolute uptake rate of strain PJ4004 in the modified assay was much higher ($190 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ dry cell mass) than in the standard assay ($14 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ dry cell mass). Both the increase in temperature (30°C compared to room temperature) and the higher free-energy state of the cells (compare the steady-state [ATP]/[ADP] ratio of 11 to that shown in Fig. 1) may have contributed to the increase; this was another reason for using the modified uptake assay procedure.

In Fig. 2, the time course of MeGlc uptake is shown for wild-type cells (with a high free-energy state) and PJ4002 cells grown in the absence of IPTG (with a lower free-energy state). The figure shows how the uptake rate was calculated by linear regression. In Fig. 2, all data points for each of the triplicate measurements are indicated separately. The individual measurements of one culture agreed well, and the linearity among the

Table 2. Intracellular ATP and ADP levels and initial MeGlc uptake rates obtained with the modified assay procedure. Cells were grown as described with the indicated IPTG concentrations in the growth medium. Transport assays were performed according to the modified method, as described in the text. $n = 3$ for both the [ATP]/[ADP] and the uptake measurements, unless indicated otherwise. Initial uptake rates were normalised with respect to the $190 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ dry cell mass observed in the wild-type strain PJ4004. Data reflect averages $\pm$ standard error of the mean.

Strain	[IPTG]	[ATP]	[ADP]	[ATP]/[ADP]	Relative MeGlc uptake
	μM	mM			
PJ4004	0	4.9 ± 0.1	0.47 ± 0.02	10.6 ± 0.5	1.00 ± 0.02
PJ4002	0	3.8 ± 0.4	0.87 ± 0.07	4.3 ± 0.1	0.79 ± 0.01
PJ4002	20	5.6 ± 0.2	0.46 ± 0.01	12.3 ± 0.2	1.10 ± 0.06^a

^a $n = 2$.

Fig. 2. MeGlc uptake for high and low cellular energy states. The graph shows the total accumulated MeGlc as a function of time for the wild-type strain (PJ4004) (closed symbols and solid lines) and the strain PJ4002 grown without IPTG (open symbols and dotted lines). Internal [MeGlc] was normalised with respect to dry cell mass. The traces for each of the triplicate determinations are shown. The lines represent a linear regression on the first four points (including zero). These points lie in the linear range and were used to calculate the initial uptake rates.

first few points was high, so that linear regression could be used as a good approximation to calculate the uptake rate.

Growth of strain PJ4002 in the absence of IPTG still resulted in a moderately high [ATP]/[ADP] ratio of 4.3, and the decrease in the initial MeGlc uptake rate, compared to the wild type, was also relatively small (Table 2). To decrease the levels of the H^+ -ATPase even further, we used strain PJ4000. The chromosomal *atp* genes in this strain are under the control of the *lacUV5p* promoter, which is more strongly repressed by the LacI repressor protein than the *tacI*p promoter of strain PJ4002. Consequently, the strain only expresses the H^+ -ATPase when the culture is induced with IPTG [13]. Like an *atp* deletion mutant, this strain will not grow on succinate when uninduced. In principle, it should be possible to fine-tune the H^+ -ATPase expression in PJ4000 to achieve even lower levels than in strain PJ4002 grown without IPTG. Under these conditions, the PJ4000 cells should also have lower steady-state [ATP]/[ADP] ratios. However, the growth rate of PJ4002 without IPTG (containing $\pm 20\%$ of the wild-type level of H^+ -ATPase) was already very low (generation time ≈ 4 h), and therefore steady-state experiments with strain PJ4000 at even lower growth rates would give a high probability of fast growing mutants (because PJ4000 cells need only to inactivate the *lacI* repressor gene to grow at the wild-type growth rate). Instead, we performed an experiment where the PJ4000 culture was pre-induced fully with IPTG to express the *atp* operon at the wild-type level, and diluted into growth medium without IPTG. This resulted in a steady decrease in growth rate (data not shown) as the available H^+ -ATPases were diluted into the growing cell population because no new enzymes were synthesised. The [ATP]/[ADP] ratio in these cells decreased to levels below 1, and the initial MeGlc uptake rate decreased concomitantly (Fig. 3). The cells virtually stopped growing when an estimated 2% of the H^+ -ATPases remained (compared to the pre-culture).

The cellular energy state of the wild-type cells (PJ4004) was also varied by adding potassium cyanide, an inhibitor of the respiratory chain, or dinitrophenol, an uncoupler of oxidative phosphorylation. The aim of this experiment was to verify that the above variation of MeGlc uptake rate with H^+ -ATPase concentration reflected a dependence of phosphotransferase system activity on free-energy state rather than a more direct depen-

Fig. 3. Initial phosphotransferase uptake activity and [ATP]/[ADP] ratios upon dilution of the H^+ -ATPase during growth. A pre-culture of strain PJ4000 was grown in the presence of 100 μ M IPTG, and different amounts were diluted into growth medium without IPTG. Thus the cultures contained different amounts of H^+ -ATPase when they reached a suitable absorbance for the uptake assay. Each point on the graph represents the average of a triple measurement for both the uptake rate and the [ATP]/[ADP] ratio. Error bars indicate the standard deviations of the measurements. The relative [H^+ -ATPase] was calculated by determining the increase in cell mass after dilution of the pre-culture. Uptake rates were normalised with respect to the 190 $\text{nmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ dry cell mass observed in the wild-type strain, PJ4004 (see also Table 2).

dence on H^+ -ATPase concentration. The addition of an inhibitor to a steadily growing cell culture will cause changes in metabolic variables. To be able to correlate phosphotransferase system uptake rates with the [ATP]/[ADP] ratio quantitatively, we therefore preincubated with inhibitor for 10 min and verified that the [ATP]/[ADP] ratio did not change significantly during the course of the subsequent experiment. We used two different concentrations of each inhibitor. Both inhibitors lowered the [ATP]/[ADP] ratio in the cells, and this was associated with a concomitant decrease in the initial phosphotransferase uptake activity in all cases (Table 3). Neither the inhibition of uptake nor the decrease in [ATP]/[ADP] ratio was severe when the lower concentration of either inhibitor was used.

We next investigated whether the variation of phosphotransferase system transport with [ATP]/[ADP] depended on the way in which the energy state was varied. Fig. 4 shows the combined results, obtained by varying the cellular free-energy state with different methods. The correlation between MeGlc uptake and [ATP]/[ADP] ratio was the same, regardless of whether the [H^+ -ATPase] was changed by steady-state gene expression or dilution. When the uncoupler dinitrophenol was added to wild-type cells, the data points on the correlation graph were on the same line as those from growing cells. Only when cyanide was used to inhibit respiration, was the initial uptake rate slightly higher for a certain [ATP]/[ADP] ratio than for the other methods. The data points for the steady-state cultures, the dilution experiment, and the dinitrophenol experiment all showed a linear trend for the dependence of initial uptake rate on the logarithm of the [ATP]/[ADP] ratio.

DISCUSSION

The results presented in this study show that changes in the cellular free-energy state, as reflected in the [ATP]/[ADP] ratio, were associated with clear effects on sugar uptake by the phosphotransferase system. In all cases studied, a lower [ATP]/[ADP] ratio resulted in a lower initial MeGlc uptake rate. The decrease

Table 3. Effects of cyanide and dinitrophenol on the cellular free-energy state and phosphotransferase system uptake. Steadily growing wild-type cells (PJ4004) were incubated with specified concentrations of inhibitor for 10 min, after which alternate ATP samples were taken and uptake assays were performed in quick succession. $n = 2$ for both the [ATP]/[ADP] and the uptake measurements, unless indicated otherwise. Uptake rates were again normalised with respect to the wild-type rate in the absence of inhibitor (value: $205 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ dry cell mass; compare Table 2). Data reflect averages $\pm$ standard error of the mean.

Inhibitor	Concentration	[ATP]	[ADP]	[ATP]/[ADP]	Relative MeGlc uptake
	μM	mM			
None	—	4.2 ± 0.1^a	0.37 ± 0.07^a	12.0 ± 2.1^a	1.00 ± 0.04
Dinitrophenol	1000	0.67^b	1.5^b	0.44^b	0.21 ± 0.03
	300	3.5 ± 0.1	0.62 ± 0.06	5.8 ± 0.6	0.83 ± 0.004
KCN	300	0.45 ± 0.10	1.3 ± 0.1	0.35 ± 0.04	0.38 ± 0.04
	70	2.0 ± 0.02	1.2 ± 0.1	1.7 ± 0.1	0.75 ± 0.03

^a $n = 3$.

^b $n = 1$.

Fig. 4. Correlation of phosphotransferase uptake activity and [ATP]/[ADP] ratio when the cellular energy state was changed by means of different methods. The graph shows the combined data from all the described experiments with the modified uptake assay method. The uptake rates were normalised with respect to the wild-type strain PJ4004 which was included as a reference in each experiment. Absolute uptake rates can be found in Tables 2 and 3. Error bars indicate the standard deviations of both the [ATP]/[ADP] and the uptake measurements.

was observed both upon changing *atp* gene expression and adding metabolic inhibitors. This suggests that the change in phosphotransferase activity was indeed the result of a lower cellular energy state rather than a more direct effect of H^+ -ATPase gene expression, as the decrease in phosphotransferase activity did not depend on the method by which the cellular energy state was varied.

Since we were interested in phosphotransferase activity under steady-state free-energy conditions, we paid special attention to the phosphotransferase system uptake assay. During the standard uptake assay, cells are chilled, washed, and concentrated in cold medium without substrate. Prior to the uptake assay, they are aerated for 3 min at room temperature, still in the absence of substrate. We found that this procedure led to a substantial decrease in [ATP]/[ADP] ratio (Fig. 1). The phosphotransferase system uptake assay was therefore modified to work under steady-state growth conditions and steady [ATP]/[ADP] ratios, even after MeGlc had been added. Significantly, the uptake rates were about 14 times higher with the improved assay method than with the standard method. This difference may be derived partially from a lower free-energy state during the standard assay

procedure (the uptake rate decreased by a factor of four when the [ATP]/[ADP] ratio was lowered tenfold; compare Fig. 4) and partially from the higher temperature in the modified assay procedure (30°C compared to room temperature).

The experiments were performed with succinate as the carbon source to achieve greater variation in the steady-state [ATP]/[ADP] ratios when the expression of the H^+ -ATPase was changed, as virtually all ATP synthesis must proceed via oxidative phosphorylation under these conditions. When cells are grown on a fermentable substrate (such as all growth substrates transported by the phosphotransferase system), additional ATP is formed during glycolysis, and even *atp* mutants have a relatively high [ATP]/[ADP] ratio [12]. Changing the gene expression of the H^+ -ATPase will hardly affect the cellular energy state under these conditions.

The relationship between initial phosphotransferase uptake rate and the logarithm of the [ATP]/[ADP] ratio, as observed in Fig. 4, is not far from linear. This may point to the quasi-linear nonequilibrium thermodynamic flow-force relationship expected on kinetic grounds [18]. The initial phosphotransferase uptake rate can then be defined as the flow that varies linearly with a thermodynamic force. The phosphorylation potential, ΔG_p , is a linear function of $\ln [\text{ATP}]/([\text{ADP}] \cdot [\text{P}_i])$, and, if the concentration of inorganic phosphate is assumed to be constant (phosphate is present in excess in the growth medium of the batch culture), ΔG_p should vary linearly with the logarithm of the [ATP]/[ADP] ratio. In this analysis, the phosphorylation potential behaves as though it were the driving force behind the phosphotransferase-system-mediated uptake. This is, of course, an empiric interpretation, but the observation that the data points for the dinitrophenol titration correlate with the linear trend of those from the experiments where *atp* gene expression was changed, suggests that the [ATP]/[ADP] ratio, an important thermodynamic driving force, is a key factor in the regulation of phosphotransferase uptake activity.

When using cyanide to inhibit respiration, the somewhat higher phosphotransferase uptake rate for a certain [ATP]/[ADP] ratio, compared to the other experiments, cannot be explained by the above interpretation alone, as both cyanide and dinitrophenol should, in principle, lower the intracellular [ATP]/[ADP] ratio by decreasing the proton gradient across the cell membrane. As cyanide also inhibits respiration, the result points to an additional effect of cyanide on phosphotransferase activity other than lowering the cellular energy state. Cyanide can react with pyruvate, for example, by nucleophilic attack on the carbonyl C

atom, forming a cyanohydrin [19], and this could possibly affect the phosphotransferase uptake rate by decreasing the concentration of intracellular free pyruvate.

The results in this study should also be viewed in the context of previous observations on the effects of metabolic activity and inhibitors of respiration on phosphotransferase system uptake [2, 3, 5, 9, 10]. As pointed out in the Introduction section, contradictory results have been reported: uncouplers and respiratory inhibitors could *stimulate* phosphotransferase-system-mediated uptake or reverse the inhibition caused by oxidisable substrates under some conditions, but under other conditions uncouplers could *inhibit* MeGlc uptake. Since these earlier studies did not report the effects on the cellular energy state and because they used differently defined experimental conditions, it is difficult to compare our data directly to the results from these earlier experiments.

Reider et al. [6] reported that the addition of uncouplers stimulated phosphotransferase system uptake activity in aerobic cells only. In addition, when the cell membrane was energised by respiration, the phosphotransferase-system-mediated uptake in membrane vesicles was inhibited; this inhibition could be reversed by the addition of cyanide [6]. The inhibition by respiration was more pronounced in *atp* mutants, although ATP levels remained unchanged [4]. Our reported inhibition of the phosphotransferase activity when lowering the expression of the *atp* operon (Table 2 and Fig. 3) may, at least in part, be the result of a proposed [6] direct effect of the membrane potential. Decreasing the $[H^+ - ATPase]$ will also decrease the proton flux used for ATP synthesis and stimulate respiration [17], resulting in an increased proton gradient across the cell membrane and hence an increased membrane potential [12], which could possibly inhibit phosphotransferase activity directly [6]. The results obtained with dinitrophenol (Table 3, Fig. 4), however, do not agree with this interpretation because the membrane potential is dissipated and phosphotransferase uptake activity is still inhibited. The correlation of phosphotransferase uptake activity and $[ATP]/[ADP]$ ratio for the dinitrophenol and gene expression experiments, even when the membrane potential was only dissipated partially by a low concentration (300 μM) of dinitrophenol, suggests that direct effects of the membrane potential on phosphotransferase activity did not play the major role in our experiments.

Our observation of decreased phosphotransferase activity upon addition of a protonophore is more in agreement with results of Hoffee and Englesberg [3] who showed that uncouplers could inhibit phosphotransferase system uptake, albeit partially, in the absence of an oxidisable substrate. Haguénauer and Képès [8] reported complete inhibition of MeGlc uptake as a result of the combined action of azide and fluoride; their result is consistent with our data. Furthermore, Klein and Boyer [7] reported a 60% decrease in the steady-state level of MeGlc accumulation when the cells were incubated with high arsenate and low phosphate, which decreased intracellular phosphoenolpyruvate levels.

As phosphoenolpyruvate is the phosphoryl donor for the phosphotransferase-system-mediated carbohydrate uptake, the question arises as to whether the effects of changes in the cellular free-energy state on phosphotransferase activity are mediated by changes in phosphoenolpyruvate concentration. In preliminary experiments, we have determined that intracellular phosphoenolpyruvate levels increased when the $[H^+ - ATPase]$ was lowered, whereas the addition of dinitrophenol led to a decrease in phosphoenolpyruvate levels (Rohwer, J. M. and Shinohara, Y., unpublished results). In both cases, however, initial phosphotransferase system uptake rates were inhibited. This shows that the cellular energy state as reflected in the $[ATP]/[ADP]$ ratio

and not the intracellular phosphoenolpyruvate level is important for the regulation of phosphotransferase activity.

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