Control of Glycolytic Dynamics by Hexose Transport in *Saccharomyces cerevisiae*

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ABSTRACT It is becoming accepted that steady-state fluxes are not necessarily controlled by single rate-limiting steps. This leaves open the issue whether cellular dynamics are controlled by single pacemaker enzymes, as has often been proposed. This paper shows that yeast sugar transport has substantial but not complete control of the frequency of glycolytic oscillations. Addition of maltose, a competitive inhibitor of glucose transport, reduced both average glucose consumption flux and frequency of glycolytic oscillations. Assuming a single kinetic component and a symmetrical carrier, a frequency control coefficient of between 0.4 and 0.6 and an average-flux control coefficient of between 0.6 and 0.9 were calculated for hexose transport activity. In a second approach, mannose was used as the carbon and free-energy source, and the dependencies on the extracellular mannose concentration of the transport activity, of the frequency of oscillations, and of the average flux were compared. In this case the frequency control coefficient and the average-flux control coefficient of hexose transport activity amounted to 0.7 and 0.9, respectively. From these results, we conclude that 1) transport is highly important for the dynamics of glycolysis, 2) most but not all control resides in glucose transport, and 3) there should at least be one step other than transport with substantial control.

INTRODUCTION

The previous century has witnessed a surge in the understanding of the molecular biology underlying the functioning of intact cells. This has culminated in the availability of complete genome sequences of unicellular and multicellular organisms, such as that of the yeast *Saccharomyces cerevisiae* (Goffeau, 1997) and of the fruit fly (Adams, 2000). The understanding of the physiology of living cells in terms of their molecular properties has begun to lag considerably behind the understanding of DNA sequences. In view of old biochemistry paradigms this seems disappointing; the secrets of the functioning of the living organisms should reside in the proteins and their genes, and in fact only in a few of these. After all, for each physiological process there was only one rate-limiting step. That step could be identified as the enzyme that was farthest removed from equilibrium. Studying and understanding the enzyme catalyzing that step, its regulation by regulatory molecules such as citrate and cAMP, and the regulation of the gene encoding that enzyme should suffice for understanding that physiological process. Experimental data have shown that the classical biochemistry paradigms are indeed too simplistic for a number of key processes for living cells. In mitochondrial oxidative phosphorylation, cytochrome oxidase is the farthest from equilibrium. Yet, it is not the rate-limiting step for that process (Groen et al., 1982). In yeast glycolysis, phosphofructokinase has long been considered the rate-limiting step, because it is the farthest from equilibrium and strongly regulated. Yet its overexpression did not enhance glycolytic flux substantially (Schaaff et al., 1989; Davies and Brindle, 1992). If flux control is at all concentrated at a single step in that pathway, then that step may be glucose transport, both in yeast (Ye et al., 1999) and in *Trypanosoma brucei* (Bakker et al., 1999). It is more likely, however, that the control is distributed over a number of steps in glycolysis (Bakker et al., 1997).

Accepting that steady-state flux is not controlled by the classical rate-limiting step, is that at all relevant for the more dynamic phenomena of the living cell? Indeed, although phosphofructokinase does not control steady flux, it has certainly been proposed to set the pace for yeast glycolytic oscillations, perhaps the best characterized biological dynamic system (Chance et al., 1964; Ghosh et al., 1971; Hess and Boiteux, 1973). In other words, it is becoming clear that transport rather than phosphofructokinase controls steady-state glycolytic flux, but how about oscillations? This is the issue addressed by this paper.

Yeast cells can reproducibly exhibit sustained glycolytic oscillations of hexose phosphates, adenine nucleotides, and the redox couple NADH/NAD^+ after addition of glucose and inhibition of respiration (Chance et al., 1964; Pye and Chance, 1966; Richard et al., 1993). Thanks to synchronization of the oscillations of the individual cells (Richard et al., 1994, 1996a; Bier et al., 2000; Wolf and Heinrich, 2000) the oscillations can be monitored macroscopically by measuring NADH fluorescence of the population of cells.

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Recently the control exerted by the glucose transporter on the steady-state flux in trypanosomes was measured by use of a competitive inhibitor (Bakker et al., 1999). We here adapt this approach to the control of oscillations in yeast, as we gradually inhibited the activity of glucose transport by titration with maltose. We report that there is substantial although not complete control of glycolytic oscillations in hexose transport, so that phosphofructokinase cannot be the oscillophore.

MATERIALS AND METHODS

Materials

Yeast nitrogen base (YNB) without amino acids was purchased from Difco (Detroit, MI). Glucose was obtained from Duchefa (Helsinki, Finland) (when used as carbon and free energy source in the medium) or from Sigma Chemical Co. (St. Louis, MO) (when used in the glucose transport assays). D-[U-^{14}C]glucose and D- [U-^{14}C]mannose were obtained from Amersham International (Arlington Heights, IL). Enzymes were purchased from Boehringer (Mannheim, Germany). Glass microfiber filters (GF/C) were obtained from Whatman (Kent, UK). Liquid scintillation fluid was purchased from Packard (Meriden, CT). Other reagents were obtained from Merck (Darmstadt, Germany), Sigma, or Fluka (Milwaukee, WI) and were of analytical grade or higher.

Strain and growth conditions

The yeast *Saccharomyces cerevisiae* (X2180, diploid strain, mal$^-$) was grown under semi-aerobic conditions at 30°C on a rotary shaker in medium containing glucose (10 g L^{-1}) or mannose (10 g L^{-1}), YNB (6.7 g L^{-1}), and phthalic acid (100 mM) at pH 5.0 (KOH). Cells were harvested at the diauxic shift, i.e., when the sugar source was exhausted, washed twice with 100 mM potassium phosphate, pH 6.8 (centrifugation, 5 min at 5000 rpm, MSE-Europa 24M), and resuspended in the same buffer. After starvation of the suspension for 2 h at 30°C on a rotary shaker, the cells were again pelleted and resuspended in the same buffer to a protein concentration (Lowry et al., 1951) of approximately 4 g L^{-1} and kept on ice until further use. For glucose-grown cells a glucose stick (Glukotest, Boehringer Mannheim) was used to time the diauxic shift. For mannose-grown cells, a growth curve was constructed to determine the optical density (OD_{600}) at which the mannose had been depleted from the medium.

Oscillations

In a thermostatically controlled cuvette at 25°C (glucose-induced oscillations) or 20°C (mannose-induced oscillations) yeast cells were incubated, and glucose or mannose was added to a final concentration of 20 mM. Cyanide (final concentration of 4 mM) was added 4 min after the addition of the hexose. In the case of maltose inhibition, maltose was added 2 min after the addition of glucose. The final concentration of maltose ranged from 20 to 100 mM. The oscillations were monitored by measuring NADH fluorescence (338-nm excitation, 456-nm emission). The frequency was determined as the reciprocal of the time interval between subsequent maxima.

Hexose consumption flux

When measuring the average glucose consumption flux, yeast cells were incubated under the same conditions as in the oscillation experiments. Samples were taken every 5 min for 30 min through a Dynagard filter (0.2 μm). The filtrate was diluted and analyzed for glucose by an NADP-linked enzymatic assay as described by Bergmeyer (1974) on an automated analyzer (COBAS, Roche, Basel, Switzerland). When measuring the average mannose consumption flux, samples were quenched in trichloroacetic acid (TCA, 5% w/v final concentration), vortexed, and put on ice. After neutralization with K_2CO_3 and centrifugation, the supernatant was diluted and analyzed for mannose. The glucose assay was modified by adding phosphoglucose isomerase and phosphomannose isomerase. To estimate the mannose consumption flux at each mannose concentration, the tangent in each point of the time course of the mannose concentration was computed by a cubic spline algorithm.

Hexose transport

Hexose transport was measured according to Walsh et al. (1994). In glucose-grown cells, glucose transport was measured in the absence and presence of 100 mM maltose at 25°C, the two sugars being added simultaneously. In mannose-grown cells, glucose transport and mannose transport were measured at 20°C.

Control analysis

The control coefficient is defined as:

$$C_i^{X_j} \equiv \left(\frac{\partial \ln X_j}{\partial \ln p}\right) \Big/ \left(\frac{\partial \ln v_i}{\partial \ln p}\right)_X, \qquad (1)$$

where X_j is the controlled variable, v_i is the activity of the controlling enzyme, and p is a parameter that specifically affects v_i (Schuster and Heinrich, 1992). To determine the flux control coefficient and the frequency control coefficient of hexose transport, the activity of hexose transport v_i was modulated. In one approach, maltose, a competitive inhibitor of glucose transport, was titrated to oscillating cell suspensions. The effects on the glucose consumption flux and on the frequency of the glycolytic oscillations were measured. The activities of glucose transport in the presence of different maltose concentrations were calculated from measured inhibition constants and from estimated intracellular glucose concentrations. These concentrations were calculated from the difference between the kinetics of glucose transport and the glucose consumption flux (cf. Bakker et al., 1999); see the Appendix for full details of this procedure. The flux and the frequency were plotted versus the calculated activity of glucose transport on a double-logarithmic scale, and the control coefficients were determined as the slopes of the curves at 100% transport activity.

In a second approach, the activity of sugar transport was modulated by varying the extracellular sugar concentration. The responses of the average glycolytic flux and the frequency were determined. By also measuring the elasticity of the sugar transporter for mannose in transport assays, the control coefficients for both flux and frequency were calculated.

RESULTS

Modulation of glucose transport by an inhibitor (maltose)

Oscillations

Cells were grown on YNB/glucose to the point of diauxic shift, starved for 2 h, and resuspended in phosphate buffer. After addition of glucose, maltose, and cyanide, oscillations were monitored by NADH fluorescence (Fig. 1). Different maltose concentrations were added to the suspension, and

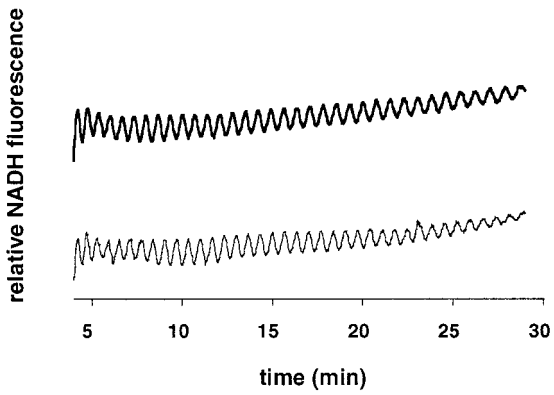

FIGURE 1 Relative NADH fluorescence during oscillations in the absence (*thin line*) and presence (*bold line*) of 100 mM maltose. Cells were grown at 30°C on glucose to diauxic shift, harvested, and starved in phosphate buffer for 2 h. Cells were subsequently resuspended to a protein concentration of approximately 4 g L^{-1}. Oscillations were induced at 25°C by adding 20 mM glucose and 4 mM cyanide to synchronize the cells. Maltose was added 2 min after the addition of glucose. Glycolytic oscillations were monitored by measuring NADH fluorescence in a fluorimeter.

the frequency of the oscillations was determined (Table 1). The frequency decreased with increasing maltose concentrations. The decrease in frequency was not due to osmotic effects, as titrating either sorbitol or galactose to the same final concentrations did not affect the frequency of the oscillations (results not shown).

Glucose consumption flux

Cells were incubated at 25°C in the presence of glucose, maltose, and cyanide at the same concentrations as in the oscillation experiments. Samples were taken at regular time intervals, and the glucose concentration in the filtrate was measured. Maltose was added to different concentrations, and the glucose consumption flux was calculated from the decrease in the glucose concentration with incubation time (Table 1). The glucose consumption flux decreased with increasing maltose concentrations. Both the absolute values of the glucose consumption flux and the percentages of inhibition differed somewhat between the different batches of cells (Table 1). Control coefficients were calculated from two experiments in which the effect of maltose on the frequency of oscillations, on the glucose consumption flux, and on the glucose transport kinetics were measured in a single batch of cells. One of these two experiments is discussed in this Results section (see Appendix for full details).

Co-response analysis

It has been speculated (Hess and Boiteux, 1973; Pye, 1973) that the frequency of the glycolytic oscillations might be determined through the pathway flux. Then one should expect flux and frequency to be proportional to each other. To evaluate the extent to which frequency and flux co-varied proportionally with each other, we quantified the co-response between frequency and flux (Hofmeyr and Cornish-Bowden, 1996). The co-response coefficient compares the extent to which two variables (e.g., X_i and X_j) are affected by some parameter change:

$$\Omega_p^{X_i, X_j} \equiv \frac{R_p^{X_i}}{R_p^{X_j}} = \frac{\partial \ln X_i}{\partial \ln X_j}, \tag{2}$$

TABLE 1 Effect of maltose on frequency and average glucose consumption flux during glycolytic oscillations
Number above each column of data is the experiment number.

Maltose concentration (mM)	Frequency (min^{-1})					Average glucose consumption flux (μmol min^{-1} mg of protein^{-1})				
	1	2	3	**4**	**5**	1	2	3	**4**	**5**
0	1.56 (100%)	1.53 (100%)	1.55 (100%)	**1.54 (100%)**	**1.48 (100%)**	0.169 (100%)	0.156 (100%)	0.193 (100%)	**0.231 (100%)**	**0.267 (100%)**
20	1.54 (98.7%)	1.50 (98.0%)	ND	**1.50 (97.4%)**	**1.45 (98.0%)**	0.166 (98.2%)	0.153 (98.1%)	0.187 (96.9%)	**ND**	**0.259 (97.0%)**
40	1.53 (98.1%)	1.49 (97.4%)	1.51 (97.4%)	**1.48 (96.1%)**	**1.42 (95.9%)**	0.160 (94.7%)	ND	0.182 (94.3%)	**0.214 (92.6%)**	**0.250 (93.8%)**
60	1.50 (96.2%)	1.46 (95.4%)	1.49 (96.1%)	**1.46 (94.8%)**	**1.40 (94.6%)**	0.159 (94.1%)	0.147 (94.2%)	0.176 (91.2%)	**0.208 (90.0%)**	**0.241 (90.5%)**
80	1.48 (94.9%)	1.45 (94.8%)	1.46 (94.2%)	**1.45 (94.2%)**	**1.37 (92.6%)**	ND	0.139 (89.1%)	0.172 (87.8%)	**0.196 (84.8%)**	**0.233 (87.1%)**
100	1.46 (93.6%)	1.43 (93.5%)	1.45 (93.5%)	**1.44 (93.5%)**	**1.36 (91.9%)**	0.152 (89.9%)	0.138 (88.5%)	0.163 (83.2%)	**0.191 (82.7%)**	**ND**

Numbers in brackets are percentages of the control (no maltose added). Oscillations were induced at 25°C by the addition of glucose and cyanide. Maltose was added 2 min after the addition of glucose. Cyanide was added 2 min after the addition of maltose. Calculation of the control coefficients was done on the basis of the fourth and fifth experiments in which frequency of glycolytic oscillations, glucose consumption flux, and glucose transport were measured for a single batch of cells.
ND, not determined.

with p being the modulated parameter. In this case X_i is the frequency of glycolytic oscillations, X_j is the glucose consumption flux, and p is the maltose concentration. In Table 2 the co-response coefficients are listed for the various experiments. The co-response coefficient ranged from 0.37 to 0.65 due to the variation in glucose consumption flux. The average co-response coefficient was 0.5 ± 0.1. This implies that flux and frequency do not vary proportionally.

TABLE 2 Co-response coefficients for the individual experiments, average co-response coefficient, and standard deviation

Experiment	$\Omega_p^{X_i,X_j}$	Average	SD
1	0.65	0.5	0.1
2	0.52		
3	0.44		
4	0.37		
5	0.55		

Glucose transport

Cells treated identically to the cells used for the oscillation and flux experiments were also used for glucose transport assays. Glucose transport was measured in the absence and presence of 100 mM maltose (Fig. 2). Glucose transport consisted of one kinetic component. Eq. 3 (see Appendix) revealed a high-affinity system (K_m 2.7 ± 0.2 mM; V_{max} 302 ± 4 nmol min^{-1} mg of $protein^{-1}$).

As the strain X2180 lacks the maltose permease, maltose is not transported into the cell (Diderich et al., 1999) and therefore it inhibits only from the outside of the carrier and breaks its (assumed) symmetry. This is reflected in two inhibition constants ($K_{i,1}$ and $K_{i,2}$). Only $K_{i,1}$ could be estimated experimentally from the transport assays in the presence of 100 mM maltose, and a value of 42 ± 3 mM was determined. A range for the possible values of $K_{i,2}$ was calculated using the extreme values that the elementary rate constants can attain (see the Appendix for details on the fitting procedures and the equations used).

To calculate the activity of glucose transport at different maltose concentrations, the relevant concentrations of substrate, product, and inhibitor at those concentrations should be known. The product, i.e., the intracellular glucose concentration, was calculated from the glucose consumption flux and the zero-*trans* influx rate, their difference being attributed to back pressure by the intracellular glucose (see Appendix). The intracellular glucose concentration was calculated to be 0.4 mM and 0.05 mM on average for the two independent experiments. For a high flux control of the glucose transporter, the rate of hexokinase should be well below V_{max}. The K_m for hexokinase with respect to intracellular glucose in de-repressed cells is 0.08 mM (Teusink et al., 2000), which implies that the rate through hexokinase should have been close to V_{max} for the first experiment and below V_{max} for the second experiment. As G6P, ATP, and ADP concentrations are not known for either experiment, we cannot calculate the rate of hexokinase. However, the measured average glycolytic flux was lower than the V_{max} of the enzyme (840 nmol min^{-1} mg of $protein^{-1}$ (Teusink et al., 2000)). This implies that the glucose transporter can have a high flux control.

The glucose transport activities, under the conditions of stationary oscillations, were calculated for the different maltose concentrations using the kinetic constants, the inhibition constants and the concentrations of extracellular glucose, intracellular glucose (see above), and maltose.

Control analysis

The glucose consumption flux and the frequency were plotted versus the calculated activity of glucose transport on a double-logarithmic scale (Figs. 3 and 4). The control coefficients were determined as the slopes of these curves at 100% activity. The average-flux control coefficient ranged from 0.7 to 0.9 and the frequency control coefficient ranged from 0.5 to 0.6, over the range of possible values of the $K_{i,2}/K_{i,1}$ ratio. These values were calculated for the experiment in which frequency, glucose consumption flux, and glucose transport were measured in a single batch of cells. In an independent duplicate experiment a flux control coefficient of 0.6 and a frequency control coefficient of 0.4 were determined. The difference in control coefficients calculated for the two extreme $K_{i,2}/K_{i,1}$ ratios was negligible in this experiment because the calculated intracellular glucose concentration was low.

The control coefficients reported here for the first approach were obtained from two independent experiments in each of which frequency, flux, and glucose transport were measured in a single batch of cells. In a third batch of cells,

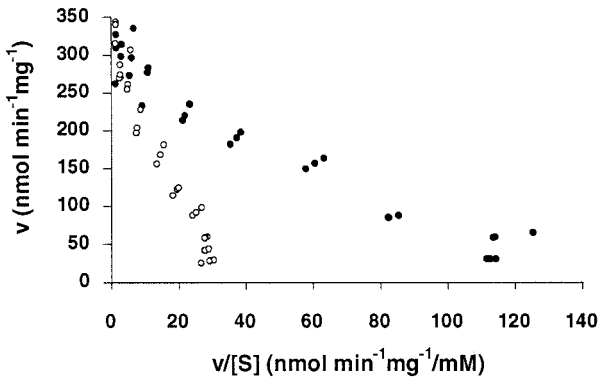

FIGURE 2 Glucose transport kinetics at 25°C in the absence (●) and presence (○) of 100 mM maltose. Cells were grown at 30°C on glucose to diauxic shift, harvested, and starved in phosphate buffer for 2 h. Cells were subsequently resuspended to a protein concentration of approximately 4 g L^{-1}. Glucose transport kinetics were measured by ^{14}C-labeled glucose uptake over a period of 5 s according to Walsh et al. (1994).

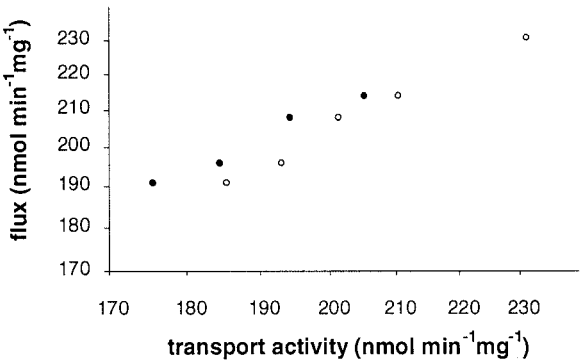

FIGURE 3 The average glucose consumption flux plotted versus the glucose transport activity at different maltose concentrations on a double-logarithmic scale for different assumed $K_{i,2}/K_{i,1}$ values. $K_{i,2}/K_{i,1} = 0.5$ (●) and $K_{i,2}/K_{i,1}$ = infinity (○). The slopes at 100% transport activity equal the control coefficients.

a two-component transport system was found (results not shown). Furthermore, the decrease in glucose consumption flux varied between the different batches of cells. These observations may reflect that around the diauxic shift the spectrum of glucose carriers in the plasma membrane changes considerably (Reifenberger et al., 1995).

Modulation of sugar transport by varying the extracellular mannose concentration

In an independent approach, sugar transport was modulated by having the cells decrease the substrate concentration. This substrate-modulation method circumvents the need for estimating an experimentally inaccessible kinetic parameter ($K_{i,2}$), as was required for the maltose inhibition approach. The high affinity of hexose transport for glucose, however, limited the concentration range in which large changes in transport activity could be achieved and stationary oscillations attained. Accordingly, mannose was used as a substrate, because the affinity of the hexose transporter(s) for mannose is much lower than for glucose. Because cells grown on glucose exhibited strongly damped oscillations with mannose as a substrate, the cells were grown on mannose and harvested at mannose depletion. The latter cells did engage in limit-cycle oscillations. If the frequency of the oscillations was controlled by sugar uptake, then one should expect a change in frequency as the sugar concentration dropped below the K_m of the carrier. This was indeed observed (Fig. 5). Under the same conditions, the transport kinetics for glucose and mannose were measured (Fig. 6). For glucose transport the K_m was 1.9 ± 0.1 mM and the V_{max} was 255 ± 7 nmol min^{-1} mg of protein^{-1}. For mannose transport the K_m was 22.5 ± 1.6 mM and the V_{max} was 174 ± 8 nmol min^{-1} mg of protein^{-1}. To estimate the mannose consumption flux at each mannose concentration, the tangent in each point of the time course of the mannose concentration was computed by a cubic spline algorithm.

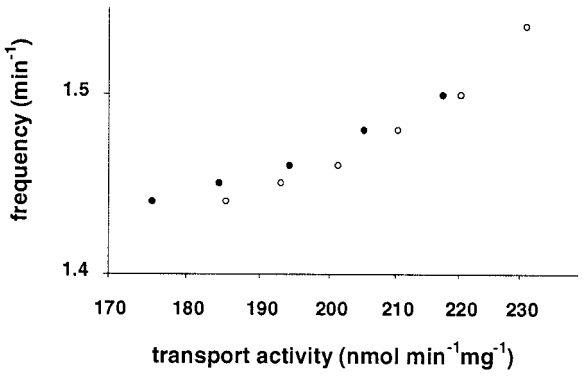

FIGURE 4 The frequency of glycolytic oscillations plotted versus the glucose transport activity at different maltose concentrations on a double-logarithmic scale for different assumed $K_{i,2}/K_{i,1}$ values. $K_{i,2}/K_{i,1} = 0.5$ (●) and $K_{i,2}/K_{i,1}$ = infinity (○). The slopes at 100% transport activity equal the control coefficients.

Surprisingly, the fitted mannose consumption flux was 2.5 times higher than the zero-*trans* influx rate (as calculated from the mannose transport kinetics and the mannose concentration), irrespective of the mannose concentration. This may be due to the activation of mannose transport activity in time that we observed (Teusink, 1999). A similar failure of zero-*trans* influx kinetics to account for the much higher rate of glucose consumption has also been noticed in cells with low-affinity transport (Teusink et al., 1998). Because also in mannose-grown cells glucose transport exhibits high-affinity kinetics (Fig. 6), high-affinity glucose carriers (such as HXT7 (Reifenberger et al., 1997)) may be subject to some activation process when faced with a poor substrate.

Assuming that the process that led to this apparent increase in mannose transport activity did not affect the elas-

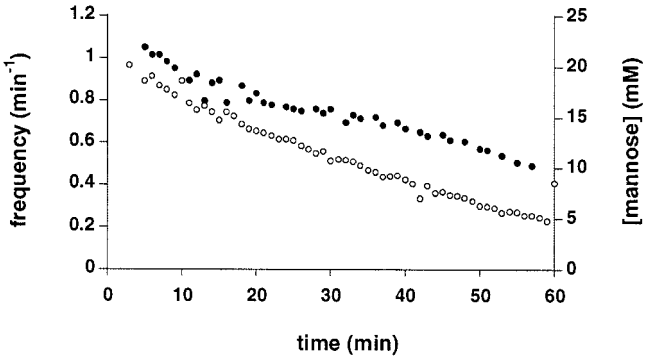

FIGURE 5 The frequency (●) and mannose concentration (○) as a function of time for mannose-induced oscillations in mannose-grown cells. Cells were grown on mannose, harvested at the diauxic shift, starved for 2 h in phosphate buffer, and resuspended to a protein concentration of 4 g L^{-1}. Oscillations were induced at 20°C by adding mannose and cyanide. The average mannose consumption flux was calculated by computing the tangent in each point of the time course of the mannose concentration, using a cubic spline algorithm.

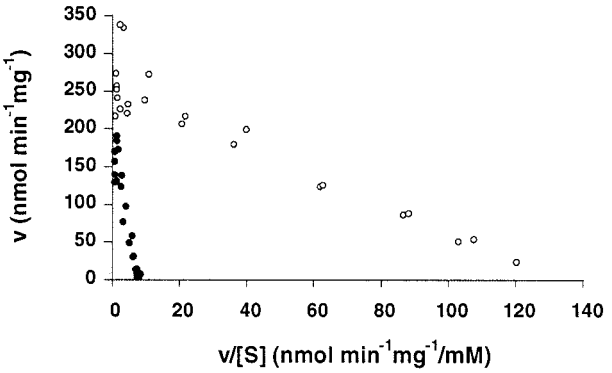

FIGURE 6 Eadie-Hofstee plot of the zero-*trans* influx kinetics of mannose transport (●) and glucose transport (○) in mannose-grown cells at 20°C. Cells were grown on mannose, harvested at the diauxic shift, starved for 2 h in phosphate buffer, and resuspended to a protein concentration of 4 g L^{-1}. Glucose and mannose uptake kinetics were measured by ^{14}C-labeled sugar uptake over a period of 5 s according to Walsh et al. (1994).

ticity of the hexose transporter with respect to the extracellular mannose concentration, the control coefficient was estimated as the slope of a double-logarithmic plot of the frequency or flux versus the zero-*trans* influx activity (Fig. 7). The frequency control coefficient estimated in this way amounted to 0.7 on average (the highest points were part of the transient phase and were not taken into account when computing the control coefficient). The analogous procedure for the mannose consumption flux led to an average-flux control coefficient of 0.9.

DISCUSSION

Control of dynamics in transport, not in oscillophore enzyme only

This paper addressed the issue whether in metabolic dynamics there needs to be a single oscillophore. Taking yeast glycolytic oscillations as the experimental model, we found that sugar transport exerted significant (i.e., >0) but not complete (i.e., <1) control on the frequency of glycolytic oscillations. This proved that not all control resides in phosphofructokinase, the proposed oscillophore; dynamic cell function is not always determined by a single, canonical, rate-limiting step.

This was shown in a quantitative, systematic manner. We determined the magnitude of the control coefficient that quantified the extent to which the sugar carrier controlled the oscillations. Two independent approaches, each with its own assumptions and growth substrate, led to the same conclusion. In the first approach, maltose was used as a competitive inhibitor of glucose transport (Diderich et al., 1999). Because the rate of the backward reaction, i.e., the glucose efflux, should also be affected by the inhibitor, the kinetic data were analyzed according to Bakker et al. (1999). Of two K_i values, one ($K_{i,1}$) could be determined. In two independent experiments, the control coefficients for frequency (0.4–0.6) did not depend more than indicated on the possible value of the other K_i. The uncertainty stemming from the absence of knowledge concerning the $K_{i,2}$, is virtually irrelevant for two questions addressed in this paper; i.e., does the glucose carrier control the oscillations at all, and does it control it fully? The answers are yes and no, respectively. This was confirmed using the second approach in which the mannose concentration varied.

Our findings in vivo are in line with experimental data on cell-free extracts (Hess and Boiteux, 1973). In that system, the transport process was substituted for by the addition of substrate at a fixed rate, the glycolytic flux being necessarily completely controlled by the pump. Plotting Hess's original data (Hess and Boiteux, 1973) on a double-logarithmic scale, we calculated in retrospect that the control of the injection rate on the frequency was 0.5 in those experiments. It is unclear how much of this was due to the artificially complete flux control by glucose influx.

Our observation that the sugar carrier does control glycolytic oscillations in vivo to a considerable extent may explain the change in frequency observed when other sugars such as fructose and mannose were used in intact cells (Kreuzberg et al., 1977), i.e., through the lower affinity and limiting rate of the transporters for those sugars (Bisson et al., 1993). Explanations focusing on inhibition of the supposed oscillophore phosphofructokinase by sugar-specific metabolic products (Kreuzberg et al., 1977; Kreuzberg, 1978) may not be necessary.

Control of average glycolytic flux largely in sugar transport

Increasing the concentrations of glycolytic enzymes did not increase the glycolytic flux (Schaaff et al., 1989; Davies and Brindle, 1992). This left hexose transport, branches from glycolysis, ATP hydrolysis, and hierarchical loops as can-

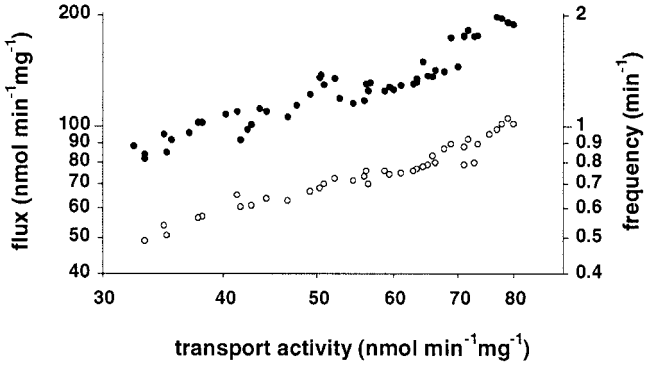

FIGURE 7 The flux (●) and frequency (○) plotted versus the zero-*trans* influx rate on a double-logarithmic scale. The zero-*trans* influx rate was calculated with the kinetics of Fig. 6 and the mannose concentrations of Fig. 5. The slopes equal the control coefficients.

didate control steps (Westerhoff et al., 2000). For *Escherichia coli*, Ruijter et al. (1991) found that glucose transport did not control flux. For *Trypanosoma brucei*, Bakker et al. (1999) observed quite a significant control in the glucose uptake step. In yeast, the issue has been difficult to address because of the multitude of glucose carriers, making molecular genetic approaches to this problem difficult. For a strain that only had the Hxt 7 transporter, the control of glycolytic flux by this carrier was close to 1 (Ye et al., 1999). However, because the control distribution is determined by the kinetic properties of the enzymes and translocators involved, the latter study did not address the situation in wild-type cells. Our present finding that in such wild-type cells control of average flux is close to 1 may suggest a way out of the paradox raised by Schaaff et al. (1989), i.e., the absence of control of glycolysis by glycolytic enzymes. The flux control may reside in sugar transport rather than in those enzymes. Our results may also suggest ways to enhance yeast glycolytic flux. In addition, it confirms (Schaaff et al., 1989; Davies and Brindle, 1992; Bakker et al., 1999) that control of glycolytic flux need not reside in phosphofructokinase or hexokinase. We point out, however, that we studied nongrowing yeast under conditions leading to glycolytic oscillations, and we determined the control on average flux under oscillatory conditions, which might be different from the control of steady-state flux.

Concluding remarks

It is becoming accepted that control of steady-state fluxes need not reside in a single rate-limiting step. The assessment of what controls cell function at steady state has become an established procedure. Understanding of the control of dynamic phenomena is still in its infancy, however, even though glycolytic oscillations have been studied for many decades (Chance et al., 1964; Ghosh et al., 1971; Hess and Boiteux, 1973; Pye and Chance, 1966; Pye, 1973; Teusink et al., 1996b; Richard et al., 1996b). The role of the different glycolytic enzymes in controlling these oscillations has never been ascertained experimentally. A number of kinetic models have been proposed in which phosphofructokinase (Goldbeter and Lefever, 1972; Goldbeter and Nicolis, 1976) or the lower part of glycolysis (Sel'kov, 1975; Dynnik and Sel'kov, 1973) was taken to be the oscillophore. Applying metabolic control analysis (MCA) to these models, however, has shown that the control on frequency and amplitude of the oscillations need not reside in one single enzyme but might well be shared by all enzymes involved in glycolysis (Bier et al., 1996; Teusink et al., 1996a). For example, Teusink et al. (1996a) compared three different models and showed that the control of glucose transport on frequency could be as high as 3 or negative. The conclusion was that, for lack of precise and validated kinetic models of the entire glycolytic pathway (Bakker et al., 1997) of yeast, calculations alone (e.g., Demin et al., 1999) could not solve this issue. Experimental determination of what controls glycolytic dynamics was called for. This has now been accomplished, and the issue settled: for cells with various histories, under various conditions, the control of the sugar transporter on the frequency of glycolytic oscillations was neither zero nor one. Because the sum of the control on frequency must be 1 (Westerhoff et al., 1990), we conclude that 1) transport is highly important for the dynamics of glycolysis, 2) most but not all control resided in glucose transport, and 3) there should at least be one step other than transport with substantial control. It is unclear whether phosphofructokinase is such a step and whether there is more than one additional step with substantial control. There may well be enzymes with negative control on the frequency (Teusink et al., 1996a).

More so than the specific numerical values of the control coefficients, this is the most important finding of this paper: control of glycolytic dynamics is subtle, i.e., not confined to a single rate-limiting step. It remains to be confirmed whether other important dynamic phenomena such as the cell cycle are also subject to such subtle control, but in view of the many factors that appear to affect cell cycling, the odds may favor this possibility (Chen et al., 2000). The implication for the number of genes that may be important when cell cycling goes astray will be clear.

APPENDIX

Glucose transport

To quantify the sensitivity of the glucose transport step for maltose we measured the glucose transport kinetics in the absence and presence of 100 mM maltose (Fig. 2). From these data the K_m and the V_{max} of the transporter as well as the K_i for maltose can be obtained. Gepasi 3.21 (Mendes, 1997) was used for the nonlinear fitting using an evolutionary programming algorithm. Zero-*trans* influx data were fitted with Eq. 3. No significant improvement in fit was obtained by including more than one transporter.

$$v = \frac{V^{+}_{max}\dfrac{[S]_{out}}{K_{m,out}}}{1 + \dfrac{[S]_{out}}{K_{m,out}}} \tag{3}$$

The following kinetic constants for the high-affinity transport system were obtained: $K_m = 2.7 \pm 0.2$ mM, $V_{max} = 302 \pm 4$ nmol min^{-1} mg of protein^{-1} and $K_m = 2.3 \pm 0.1$ mM, $V_{max} = 304 \pm 3$ nmol min^{-1} mg of protein^{-1} for the fourth and fifth experiments, respectively. Using these kinetic constants the K_i value for maltose can be estimated by fitting the following equation for competitive inhibition to the data from the transport experiments in the presence of 100 mM maltose:

$$v = \frac{V^{+}_{max}\dfrac{[S]_{out}}{K_{m,out}\left(1 + \dfrac{[I]}{K_{i,1}}\right)}}{1 + \dfrac{[S]_{out}}{K_{m,out}\left(1 + \dfrac{[I]}{K_{i,1}}\right)}} \tag{4}$$

This resulted in a $K_{i,1}$ of 42.2 ± 3.0 mM and 37.6 ± 3.3 mM for the two independent experiments

To calculate the sensitivity of the transport step for maltose, it is important to know the substrate, product, and inhibitor concentrations. The intracellular glucose concentration can be calculated from the measured zero-*trans* influx rate and the measured glucose consumption flux in the absence of maltose. The difference between this rate and the flux is assumed to be due to intracellular glucose. Using the equation

$$v = \frac{V^{+}_{max}\frac{[S]_{out}}{K_{m,out}} - V^{-}_{max}\frac{[S]_{in}}{K_{m,in}}}{1 + \frac{[S]_{out}}{K_{m,out}} + \frac{[S]_{in}}{K_{m,in}} + \alpha\frac{[S]_{out}}{K_{m,out}}\frac{[S]_{in}}{K_{m,in}}}, \quad (5)$$

the intracellular glucose concentration can be calculated. The interactive constant α depends on the relative mobility of the unbound and bound carrier (Kotyk, 1976; Teusink et al., 1998). The transport of glucose across the membrane occurs via facilitated diffusion. Assuming the transporter to be symmetrical, the K_m values for extracellular and intracellular glucose are equal. Also the V^{+}_{max} and V^{-}_{max} are assumed to be equal. The calculated intracellular glucose concentrations were 0.4 mM and 0.05 mM for the two independent experiments. Maltose is not transported into the cell and therefore it inhibits only on the outside of the carrier and breaks the symmetry of the carrier. This is reflected in two K_i values, $K_{i,1}$ and $K_{i,2}$. From the experiments presented here only $K_{i,1}$ could be estimated. (i.e., in the absence of intracellular glucose). The ratio $K_{i,2}/K_{i,1}$ can vary between 0.5 and infinity as was calculated using the elementary rate equations (Bakker et al., 1999). For the different maltose concentrations the rate of the transport step can be calculated using the formula

$$v = \frac{\frac{[S]_{out}V^{+}_{max}}{K_{m,out}\left(1+\frac{[I]}{K_{i,1}}\right)} - \frac{[S]_{in}V^{-}_{max}}{K_{m,in}\left(1+\frac{[I]}{K_{i,1}}\right)}}{1 + \frac{\left(1+\frac{[I]}{K_{i,2}}\right)[S]_{in}}{K_{m,in}\left(1+\frac{[I]}{K_{i,1}}\right)} + \frac{[S]_{out}}{K_{m,out}\left(1+\frac{[I]}{K_{i,1}}\right)} + \frac{[S]_{in}[S]_{out}\alpha}{K_{m,in}K_{m,out}\left(1+\frac{[I]}{K_{i,1}}\right)}}, \quad (6)$$

with the parameter values as determined in the transport and flux experiments. Subsequently, the derivative of v with respect to I can be calculated.

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