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Hierarchical and metabolic regulation of glucose influx in starved *Saccharomyces cerevisiae*

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Abstract

A novel method dissecting the regulation of a cellular function into direct metabolic regulation and hierarchical (e.g., gene-expression) regulation is applied to yeast starved for nitrogen or carbon. Upon nitrogen starvation glucose influx is down-regulated hierarchically. Upon carbon starvation it is down-regulated both metabolically and hierarchically. The method is expounded in terms of its implications for diverse types of regulation. It is also fine-tuned for cases where isoenzymes catalyze the flux through a single metabolic step.

Keywords: Functional genomics; Regulation; Starvation; Glucose transport

1. Introduction

Biology has changed with the advent of methods in genomics, proteomics and metabolomics. These technologies enable scientists to monitor simultaneously the concentrations of thousands of components. Moreover, they do so for various categories of compounds, such as mRNA's, proteins and metabolites. The complexity of biological processes is reflected in the resulting datasets. Transcriptome analysis has taught us that the expression of many genes at the level of mRNA is up- or down-regulated when the internal or external environment of the cell is changed. Less and less frequently, however, mRNA levels are taken to represent gene expression per se. For, changes at the transcriptome level are not always transmitted to the proteome level and perhaps not to the metabolic or physiomic level either [1,2].

Indeed, a fundamental issue is the extent to which mRNA and protein concentrations determine functional properties of the cell such as metabolic fluxes. Research on the transcriptional regulation of cellular properties has clearly dominated the recent literature, perhaps because of the relative novelty and ease of the hybridization array technology [3]. Metabolic flux can, however, be regulated simultaneously at the levels of transcription, translation and metabolism, or even at the metabolic level only. When it is regulated at either of the two latter levels, then correlating the transcriptome with function would seem to lose its meaning. All of this being recognized in principle, little has been done to deal

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with this complication. Perhaps it is not altogether clear how one should determine the relative contributions of transcription, translation and metabolism to the overall regulation of flux.

Mathematical analysis of biochemical systems has been successful in quantifying the *control* exerted by component properties upon system variables such as flux and metabolite concentrations [4–7]. These frameworks, however, do not address the question upon how living systems actually *regulate* their system properties when challenged with an environmental change. It is not always clear which are the constraints and drives governing the regulation of a cellular system, but its outcomes are often accessible to experimental determination. Ter Kuile and Westerhoff have proposed a method called 'regulation analysis', in order to disentangle quantitatively 'hierarchical' from 'metabolic' regulation of flux [2,8]. Their term 'hierarchical' refers to all processes that determine the active enzyme concentration (e.g., transcription, translation and post-transcriptional modifications), while the term 'metabolic' includes all metabolic processes that alter enzyme activity through substrate, product and effector concentrations. In a further refinement these two types of regulation can later be analyzed in more detail, e.g., to quantify transcriptional from translational control. Although regulation analysis is compatible with and complementary to metabolic control analysis [6,7] and its extension hierarchical control analysis [9], it has the special advantage of being applicable not only to small changes but also to large changes of flux. This makes it much more accessible to experimentation.

The idea is as follows. Usually enzyme rate equations are of the kind

$$v = v(e, \mathbf{X}) = f(e) \cdot g(\mathbf{X}) \tag{1}$$

in which v is the rate, e is the concentration of the enzyme and $\mathbf{X}$ is the vector of substrate, product and other effector concentrations. The important point of the above equation is that g does not depend on the enzyme concentration. This reflects the virtually universal feature that enzymes function as catalyst only, i.e., neither as substrate nor product. In logarithmic space this becomes

$$\ln v = \ln f(e) + \ln g(\mathbf{X}). \tag{2}$$

This dissects the rate equation into a term that only depends on the enzyme concentration and a term that only depends on the concentrations of metabolites and effectors. At steady-state, the pathway flux J through the enzyme equals the rate v at which the enzyme catalyses the reaction. When one wishes to ask to what extent J is regulated by the enzyme concentration and to what extent it is regulated by the metabolic term $g(\mathbf{X})$, it is useful to divide as follows:

$$1 = \frac{\Delta \ln v}{\Delta \ln J} = \frac{\Delta(\ln f(e) + \ln g(\mathbf{X}))}{\Delta \ln J} = \frac{\Delta \ln f(e)}{\Delta \ln J} + \frac{\Delta \ln g(\mathbf{X})}{\Delta \ln J} = \rho_{\mathrm{h}} + \rho_{\mathrm{m}} \tag{3}$$

in which ρ_{h} is the hierarchical regulation coefficient and ρ_{m} is the metabolic regulation coefficient. The fact that the two regulation coefficients sum to 1 is referred to as the summation theorem for the regulation of flux.

Experimentally the hierarchical regulation coefficient is the one that is more readily determined, as the function $f(e)$ usually equals the maximum enzyme rate $V_{\max}$. The hierarchical regulation coefficient then becomes

$$\rho_{\mathrm{h}} = \frac{\Delta \ln V_{\max}}{\Delta \ln J}. \tag{4}$$

Determination of ρ_{h} then depends on the possibility of measuring the $V_{\max}$ and the flux through the enzyme. This is often possible, albeit technically challenging. First, the $V_{\max}$ should be measured under physiologically relevant conditions. Secondly, when the metabolic network is complex without a single major flux routing, it may require flux analysis to resolve the intracellular fluxes [10,11]. Thirdly, when isoenzymes with different substrate affinities are active at the same time, it might seem necessary to do the analysis for each isoenzyme independently. However, in Section 4 we demonstrate that this limitation can often be overcome by a precise interpretation of the coefficients. Yet, if these considerations are made, the hierarchical regulation coefficient can be determined experimentally. As soon as the hierarchical regulation coefficient is known, the metabolic regulation coefficient follows automatically from the summation theorem.

In this paper we wish to explore the potential of regulation analysis and to apply it to the case of glycolytic flux in starved *Saccharomyces cerevisiae*. In many industrial applications of this yeast, periods of starvation occur and it is known that these influence the rate of alcoholic fermentation [12–14]. Also it is known that under starvation conditions proteins are actively degraded [15], among which glucose transporters are no exception [16].

Here we address the question to what extent a change in glucose flux due to nutrient starvation should be attributed to 'hierarchical regulation' including alterations in gene expression and degradation of the glucose transporters, and to what extent it should be attributed to changes in the interaction of the transporters with the rest of metabolism. The kinetic behavior of the glucose transporters can be described by the rate equation for a symmetric carrier [17]

$$v = V_{\max} \cdot \frac{\left(\frac{[\mathrm{Glc}]_{\mathrm{out}}}{K_{\mathrm{m,out}}} - \frac{[\mathrm{Glc}]_{\mathrm{in}}}{K_{\mathrm{m,in}}}\right)}{1 + \frac{[\mathrm{Glc}]_{\mathrm{out}}}{K_{\mathrm{m,out}}} + \frac{[\mathrm{Glc}]_{\mathrm{in}}}{K_{\mathrm{m,in}}} + \alpha \cdot \frac{[\mathrm{Glc}]_{\mathrm{out}}}{K_{\mathrm{m,out}}} \cdot \frac{[\mathrm{Glc}]_{\mathrm{in}}}{K_{\mathrm{m,in}}}} \tag{5}$$

in which $K_{m,out}$ and $K_{m,in}$ are the Michaelis–Menten constants for extra- and intracellular glucose, respectively, and α is a factor that depends on the relative mobility of the loaded and the unloaded carrier protein. Substituting this in Eq. (3) gives

$$\frac{\Delta \ln v}{\Delta \ln J} = \frac{\Delta \ln V_{max}(e)}{\Delta \ln J} + \frac{\Delta \ln g([Glc]_{out}, [Glc]_{in}, K_{m,out}, K_{m,in}, \alpha)}{\Delta \ln J} = \rho_h + \rho_m = 1. \quad (6)$$

This equation emphasizes two points. First, g is a function that describes the enzyme's interaction with the rest of metabolism, and it depends not only on the concentrations of metabolic modifiers, but also on the enzyme's affinity for them. Second, it is the cross-independence of the multipliers in the kinetic equation that allows regulation analysis to be performed over large changes.

In this study we quantified precisely the effect of either carbon or nitrogen starvation on the steady-state glucose influx as well as on the zero-*trans* rate of glucose uptake, and used these data to determine the hierarchical and metabolic regulation coefficients. We will show that nitrogen and carbon starvation give rise to different regulation of glucose flux. Furthermore we wish to use this study as an example to show that regulation analysis allows unambiguous and quantitative statements about how the cell achieves large changes of flux when it faces new conditions.

2. Materials and methods

2.1. Growth and starvations

Saccharomyces cerevisiae strain CEN-PK 113-7D (*MATa MAL2-8*^{c} *SUC2*) was grown in controlled batch cultures of 1.5 l at a stirrer speed of 800 rpm and at 30 °C in defined mineral medium containing 101 mM glucose [18]. The culture was kept at pH 5.0 by titration with 2-N KOH and aerated by flushing air at 45 l h^{-1} through the culture. Cells were harvested by centrifugation at an $OD_{600\,nm}$ of 1.0 (exponential phase). For starvation experiments, the pellets were washed with equal volumes of ice-cold growth medium lacking either glucose or ammonium, and resuspended in their corresponding medium to a cell density of 0.75% wet weight (≈1 g dry weight l^{-1}) at pH 6.0. The suspensions, of ≈300 ml, were kept in 2-l shake flasks on a rotary shaker at 30 °C and 200 rpm without pH control for 24 h. To avoid dual starvation, the growth medium lacking ammonium contained 177 mM glucose. After 24 h the glucose concentration in the supernatant of nitrogen-starved cultures was about 100 mM. It was checked, by chemical analysis, that all chemical elements present in the growth medium (N – in carbon starvation –, K, Na, Ca, Mg, Cl, SO_4, P, Fe, Mn, Zn, B, Cu, Mo) were still in excess after 24 h of starvation. For the measurement of steady-state glucose influx, the cells were harvested by centrifugation and resuspended in growth medium without a carbon source. Unstarved cells were resuspended to 3% wet weight (5 g dry weight l^{-1}), while nitrogen starved and carbon starved cells were resuspended to a density of 6% wet weight (10 and 5 g dry weight l^{-1}, respectively) and kept on ice for at most 1 h. Similarly, for the measurement of zero-*trans* influx of glucose, cells were harvested by centrifugation and resuspended in growth medium without carbon or nitrogen source, all cultures were resuspended to 7.5% wet weight (resulting in 10 g dry weight l^{-1} for unstarved and carbon-starved and 15 g dry weight l^{-1} for nitrogen-starved cells) and kept on ice for at most 1 h.

2.2. Steady-state glucose influx

Steady-state glucose influx was measured in a cell suspension kept anaerobic at 30 °C in a setup described by van Hoek et al. [19] for determination of the fermentative capacity, with the following modifications: cells were washed and resuspended in growth medium without a carbon source. The headspace was flushed with N_2 instead of with CO_2, and glucose was measured by HPLC (300 mm × 7.8 mm Ion exchange column Aminex-HPX 87H (Biorad), with 22.5 mM H_2SO_4 kept at 55 °C as eluent at the flow rate of 0.5 ml min^{-1}).

2.3. Zero-trans influx of glucose

Zero-*trans* influx of ^{14}C-radiolabelled glucose was determined in a 5-s uptake assay at 30 °C according to Walsh et al. [20], with the modifications: (1) that the uptake assay was carried out in the growth medium (see above) and (2) that the cells were aerated during preincubation at 30 °C for 4 min prior to the uptake assay. The range of glucose concentrations was between 0.25 and 225 mM. Irreversible Michaelis–Menten equations were fitted to the results by non-linear regression using SigmaPlot 2001 version 7.0 (SPSS Inc.).

3. Results

3.1. Nutrient starvation leads to a decrease of the glucose influx

The aim of our study was to quantify to what extent the decrease of glucose consumption due to starvation is regulated by expression or degradation of the transporters and to what extent it is regulated by the interaction of these transporters with the rest of metabolism. To this end we first quantified the decrease of the steady-state

glucose influx under standardized conditions of growth and starvation. *S. cerevisiae* CEN.PK 113-7D was grown in a well-aerated and pH-controlled batch culture. An aliquot of cells was harvested during exponential growth and split in three parts. One part was washed and transferred to an anaerobic vessel with fresh medium with excess of glucose (101 mM). Under those conditions (referred to as 'unstarved') the glucose flux was measured over a period of 30 min. The other two batches of cells were washed and transferred to fresh medium, lacking either ammonium (N-starved cells) or glucose (C-starved cells). After 24 h these cells were again harvested and subsequently treated as the unstarved cells had been, now to quantify the glucose influx under glucose-excess conditions. Importantly, throughout the duration of all assays, glucose concentration remained in excess. Estimations using the kinetic parameters in Table 1 predict negligible changes in glucose transport rate due to the changes in external glucose measured in this assay. The rate of glucose consumption was constant throughout the assay although in some occasions an initial lag phase of a few minutes was observed. This phenomenon proved not to be reproducible and we cannot give a causal explanation. The rate was decreased by 75% and 80%, respectively, after N- and C-starvation (Fig. 1). The difference between N- and C-starvation was not statistically significant. Protein and dry-weight measurements indicated that there was no substantial growth during the assay (30 min) in any of the conditions (not shown).

Table 1
Kinetic parameters of the irreversible Michaelis–Menten equations after fitting to the dependence of rate on glucose concentration (cf. Fig. 2)

Condition	V_{max}	K_m
Unstarved	737 ± 104	38 ± 2
N-Starved	150 ± 1	7.7 ± 2.5
C-Starved	407 ± 8	32 ± 11

V_{max} units are nmol min^{-1} mg protein^{-1} and K_m units are mM. Errors represent standard deviations based on two independent experiments with different cell batches.

3.2. Zero-trans influx of glucose

Subsequently we investigated to what extent the observed decrease of the steady-state glucose consumption flux was paralleled by a changed capacity of the glucose transporters. To quantify the zero-*trans* uptake kinetics of glucose, the uptake of radio-labeled glucose was measured during 5 s at glucose concentrations between 0.25 and 225 mM, and irreversible Michaelis–Menten equations were fitted to the results. Both types of nutrient starvation led to a decrease of glucose transport capacity. The decrease was more severe in nitrogen-starved cells (Fig. 2). During N-starvation a decrease in K_m was observed, while the affinity during carbon starvation was unchanged within statistical error (Table 1).

We then compared the steady-state glucose consumption flux to the transport capacity at 0.1 M (i.e., the concentration at which the flux had been measured, Fig. 1). In unstarved and nitrogen-starved cells the steady-state rate of glucose consumption was similar to the transport capacity, indicating that the transporter worked at maximum capacity and that product inhibition by intracellular glucose [21] was negligible, i.e., internal glucose was and remained low. In contrast, carbon-starved cells

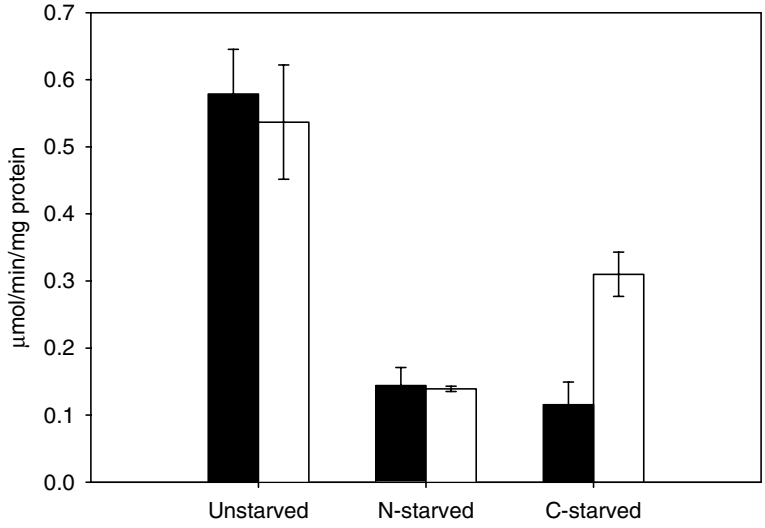

Fig. 1. Comparison of steady-state glucose consumption flux with the capacity of the glucose transporters at 0.1 M as determined by zero-*trans* influx experiments. Steady-state glucose consumption flux (black bars) and zero-*trans* influx glucose transport capacity (white bars). The error bars represent the standard deviation of two independent experiments carried out with different batches of cells.

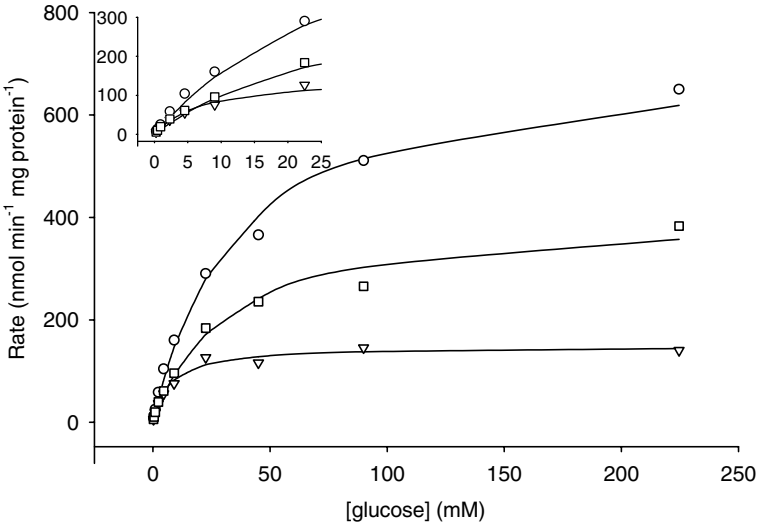

Fig. 2. Dependence of glucose uptake rate upon glucose concentration as measured in zero-*trans* influx experiments. The rate of glucose uptake at different glucose concentrations was measured for three conditions: unstarved (circles), nitrogen-starved (triangles) and carbon-starved (squares). Symbols represent averages of two independent experiments carried out with different batches of cells. Lines represent fits of irreversible Michaelis–Menten equations (for fitted parameters see Table 1). The inset illustrates the rate dependence upon glucose concentration at the lower glucose concentrations.

possessed a two- to three-fold excess glucose transport capacity compared to the actual glucose consumption. This difference is probably due to product inhibition of the glucose transporters by a relatively high internal glucose concentration in the steady-state situation. Accumulation of internal glucose can also affect the zero-*trans* influx rate by decreasing the apparent rate of glucose uptake. In order to estimate the magnitude of this underestimation, we simulated our 5-s glucose uptake assay for the carbon-starved cells, assuming that glucose accumulated in the cell without further metabolism (maximum internal glucose accumulation possible). In the simulation we used the rate equation for a symmetrical carrier ([17], Eq. (5)) with the parameters reported for carbon-starved cells (Table 1) and using an estimated cell volume of 3.75 μl per mg protein [22]. The rate of glucose consumption with unrestricted internal glucose accumulation was about 10% lower than the zero-*trans* rate; therefore we expect that the underestimation of glucose transport capacity due to product inhibition by internal glucose is at most 10%. This result seems to contradict the findings by Smits et al. [23], who studied glucose transport in a triple hexose-kinase deletion mutant and found that absence of further metabolism of glucose severely impaired the accuracy of 5-s zero-*trans* influx determinations. They also showed that this difficulty could be overcome by measuring in the much shorter time scale of 200 ms. The apparent contradiction is resolved when it is realized that the affinity of the glucose transporter in the glycerol-grown triple hexose-kinase deletion strain was found to be much higher than in our carbon-starved cells (2.1 mM compared to 32 mM). Under the assumption that the glucose carrier is symmetrical, a high-affinity transporter is much more sensitive to product inhibition by internal glucose than a low-affinity transporter. We made a similar simulation using the kinetic parameters reported by Smits et al. and found that the rate of glucose consumption with unrestricted internal glucose accumulation during 5 s was about 35% lower than the 200-ms zero-*trans* rate.

3.3. Regulation analysis of glucose influx

The hierarchical regulation coefficient ρ_h is a quotient that relates the relative change in V_{max} with the relative change in flux and its numerical value may be any real number. We have classified its possible numerical values into five distinct categories, each with a precise biochemical interpretation. Fig. 3 illustrates the biochemical interpretation of each of these five categories. In this figure, the relation between the rate and the substrate concentration is drawn for an enzyme described by irreversible Michaelis–Menten kinetics. The two curves have the same K_m, but different V_{max}-values, which correspond to a change in the amount of the enzyme. The arrows represent changes in V_{max} and/or substrate concentration that result in a change in rate. The arrows are labeled with boxed numbers that identify them as pertaining to one of the categories listed below: (the nominal numbers correspond to the arrow numbers):

1. $\rho_h = 1$ Means that the relative change of V_{max} equals that of the flux. This implies that there is no metabolic regulation. In the example of Fig. 3 this is obvious from the fact that the substrate concentration does not change (see arrow 1). In more complex

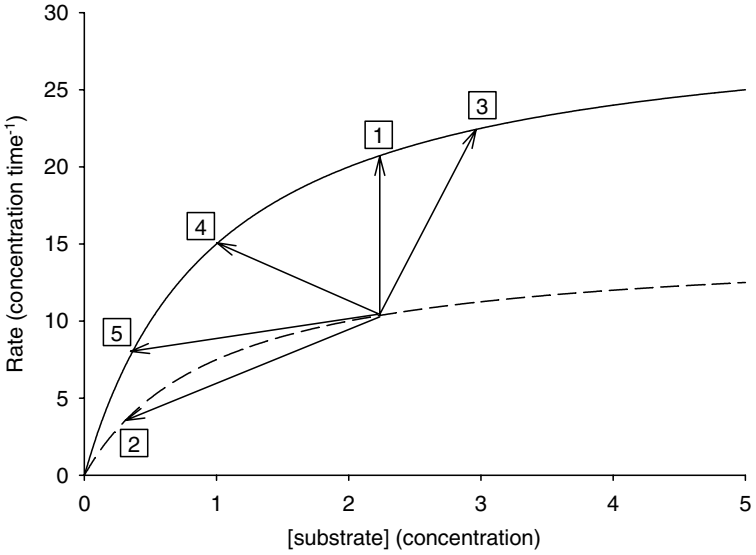

Fig. 3. Biochemical interpretations of regulation coefficients: the dependence on the substrate concentration is depicted for two enzymes described by irreversible Michaelis–Menten kinetics. Both enzymes have a K_m of 1 (concentration units). They differ in their V_{max}-values (concentration/time units), of V_{max} 30 (solid line) and V_{max} 15 (dashed line). Arrows represent changes in V_{max} and/or substrate concentration that result in a change in rate. Arrow labels correspond to categories of biochemical interpretations explained in the main text.

cases, metabolite concentrations and K_m values may change, but such that there is no net change of the function that expresses the interaction of the enzyme with the rest of metabolism. In such cases the internal changes in the composition of the metabolic function do not contribute to a change of flux and therefore we say that there is no metabolic regulation.

2. $\rho_h = 0$ Means that V_{max} remains unchanged. The decrease in flux is caused solely by a change of the metabolic function, i.e., in this example by a reduction in the substrate concentration. This is a case of exclusively metabolic regulation.
3. $0 < \rho_h < 1$ Means that the relative increase in V_{max} is smaller than the relative increase in flux. From the summation theorem (Eq. (6), Section 1) it follows that the metabolic regulation coefficient also takes a value between 0 and 1. Thus the flux is changed by both a change in V_{max} and a change in the metabolic function. In the example, the latter is achieved by an increase of the substrate concentration (Fig. 3).
4. $\rho_h > 1$ Means that the relative change in V_{max} is larger than the relative change in flux. The changes in V_{max} and substrate concentration have antagonistic effects on the flux: the increase in V_{max}, for instance due to an increase in transcription, hauls the flux to increase, the decrease in substrate concentration keeping the flux back. The net result is a change of the flux in the same direction but not quite as much as the change in V_{max}. Here hierarchical regulation is dominant and metabolic regulation homeostatic. This case is expected when an organism overexpresses a step in a pathway with a low/intermediate flux control coefficient. The substrate of that step will then decrease and the product concentration will go up, making metabolic regulation buffer away the regulation through gene expression.
5. $\rho_h < 0$ Means that the V_{max} and the flux change in opposite directions. As in category 4, the changes in V_{max} and substrate concentration have antagonistic effects on the flux. In this case, however, the flux changes in the direction of the metabolic function and therefore metabolic regulation is dominant.

To illustrate the principle, ρ_h can be represented graphically in a coordinate system with axes '$\Delta \ln flux$' and '$\Delta \ln V_{max}$' (Fig. 4). Each point in this plot represents a change in V_{max} and flux through a specific enzyme that results from whatever change in the internal or external environment of the cell. The slope of the line through the data-point and the origin equals ρ_h. The numbers in Fig. 4 correspond to the different categories distinguished above. This illustration highlights the possibility of positive as well as negative changes in flux and V_{max}, and any combination thereof. It also introduces the possibility of representing simultaneously the regulation of different enzymatic reactions in response to a given perturbation, and/or the regulation of a single enzymatic reaction in response to different perturbations, facilitating comparison and classification.

In order to apply regulation analysis to our experimental results, the relative change in glucose transport capacity ($\Delta \ln V_{max}$) was divided by the relative change in glucose consumption ($\Delta \ln flux$), during either type of nutrient starvation. The relative change in glucose consumption during nitrogen starvation was similar to the relative change in the capacity of the glucose transport-

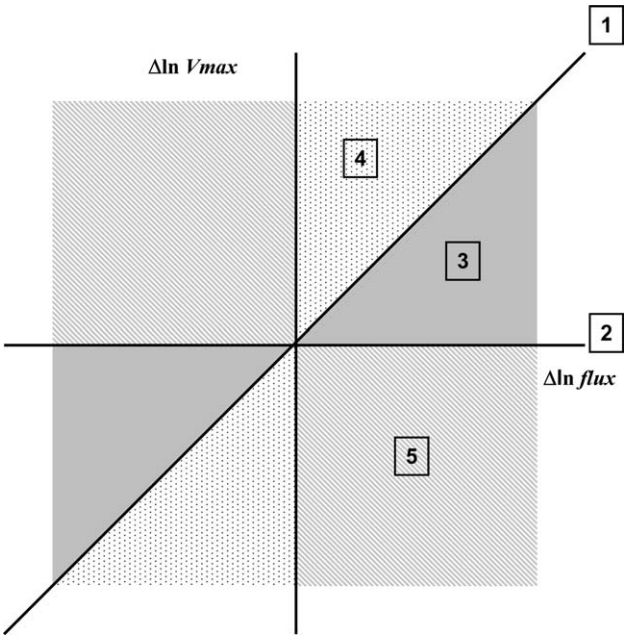

Fig. 4. Graphical representation of the different modes of steady-state flux through an enzyme. This coordinate system with axes corresponding to the relative changes in flux and V_{max} allows a graphical representation of any process in which these properties are changed in a common plot. Furthermore, the slope of the line connecting any data point to the origin represents the metabolic regulation coefficient, ρ_h, of the represented process. The plot includes a diagonal line with slope 1 (referred to by a box 1) that represents all processes in which ρ_h is 1, i.e., where regulation is completely hierarchical. All other possible slopes are grouped in the differently shaded areas and their biochemical interpretation is explained in the main text.

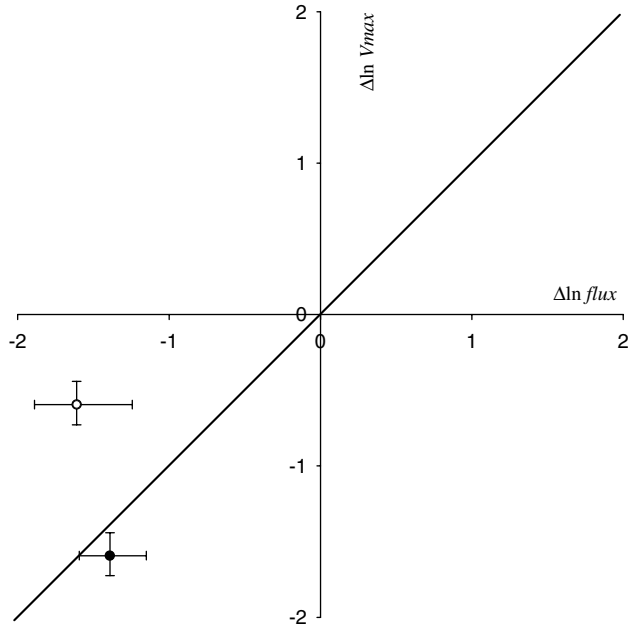

Fig. 5. Regulation of glucose flux by glucose transport capacity upon starvation. A graphical representation of ρ_h in a coordinate system with 'Δln *flux*' and 'Δln V_{max}' as axes. Nitrogen (closed circle) and carbon (open circle) starvations are represented, error bars correspond to the standard deviation. The diagonal line with slope 1 represents all processes in which ρ_h is 1. The nitrogen-starvation results lie somewhat below the diagonal line in the area corresponding to slopes exceeding one and denoted in Fig. 1 as category 4 (antagonistically regulated by hierarchical an metabolic processes and dominated by the former). Carbon starvation results populate the area corresponding to category 3, this is of slopes between 0 and 1 (simultaneous regulation by hierarchical and metabolic processes).

ers, with a ρ_h of 1.1 ± 0.2 (dimensionless, error indicates standard deviation). This implies that regulation is classified best in category 1 (Figs. 4 and 5, pure V_{max} regulation). However, it is possible that there is a small contribution of metabolic regulation, counteracting the V_{max} regulation ($\rho_m = 1 - 1.1 \pm 0.2 = -0.1 \pm 0.2$). In contrast, in carbon starvation the relative change in glucose consumption exceeded the relative change in glucose transport capacity, with resulting ρ_h of 0.4 ± 0.1. This meant that the change in flux was brought about both by the change in capacity and by the interaction of the enzyme with the rest of metabolism. From the summation theorem it follows that the metabolic regulation coefficient ρ_m is $1 - 0.4 \pm 0.1 = 0.6 \pm 0.1$in this case. This is a typical example of cooperative regulation (category 3, Figs. 4 and 5), in which both the change in V_{max} and the interaction of the enzyme with the rest of metabolism contribute positively to the change of flux.

4. Discussion

Regulation analysis was devised to disentangle the relative contributions of 'gene-expression' and 'metabolic' processes to the regulation of functional processes in the living cell [2,8]. In this paper we have applied regulation analysis to the regulation of glucose flux through glucose transporters during nutrient starvation. In this section we will, based on our experimental findings, discuss the scope and limitations of regulation analysis.

Nutrient starvation has been reported previously to result in a decreased glucose consumption, which is accompanied by a decrease in glucose transport activity ([13,14], Fig. 1), but the correlation between flux and transport activity was not unequivocal. Authors have expressed this in qualitative statements such as: ''the reduction in flux is partly explained by the reduction in transport capacity'' [13] or ''the changes in flux were not reflected by the changes in glucose transport capacity'' [14]. These statements were true, yet vague. Unambiguous and quantitative statements are to be preferred and regulation analysis has been proposed as a method to accomplish this [8].

We studied the effects of carbon and nitrogen starvation on glucose consumption flux and on glucose transport capacity and its detailed kinetics. Consistently with previous studies, we found that both glucose consumption and transport capacity are decreased during

starvation. However, the decrease of glucose transport relative to glucose flux was very different in the two types of starvation (Fig. 1). We applied regulation analysis to our data and found that during nitrogen starvation the ρ_h was 1.1 ± 0.2, meaning that the flux was regulated mostly hierarchically, with at most a small and antagonistic contribution by metabolism ($\rho_m = -0.1 \pm 0.2$). A minor contribution of the change in the metabolic function to the regulation of the flux does not imply that the changes in the individual variables of this function are small. Instead, it means that these changes taken together have little *overall* effect on the flux and thereby hardly contribute to its regulation, i.e., total metabolic regulation is small.

In the case of carbon starvation we found a ρ_h of 0.4 ± 0.1. From the summation theorem it followed that the metabolic regulation coefficient (ρ_m) was 0.6 ± 0.1, meaning that the change in flux was brought about for some 40% by hierarchical and some 60% by metabolic regulation. We have now, for the first time, made unambiguous and quantitative statements regarding the regulation of the glucose consumption flux by changes in the capacity of its transporters. Moreover we have reinforced a novel method in which this can be done for many other cases and systems as well.

It must be acknowledged that the measurement of the glucose flux and that of the glucose uptake rate are at different time scales (30 min versus 5 s). It cannot be excluded that the transporter kinetics change throughout the flux assay. This will be the subject of further studies.

The detailed kinetic study revealed that during nitrogen starvation not only the transport capacity but also the apparent K_m of the transporters was decreased (Fig. 2 and Table 1). Changes in the apparent affinity of transporters when cells are challenged by an environmental change are well known [20] and they can be ascribed to changes in the isoenzyme distribution of the transporters [24]. *S. cerevisiae* contains 17 genes homologous to genes encoding glucose transporters. Seven of these genes (*HXT1-7*) and *GAL2* are considered as the most important glucose transporter genes, since deletion of these genes is sufficient to abolish growth on glucose [25–27]. Differential expression of the *HXT* genes has been shown to be an important mechanism to modulate the apparent overall affinity of glucose transport for its substrate, extracellular glucose [24].

In our own study we found that the overall kinetics of zero-*trans* glucose influx could be described by a single Michaelis–Menten equation. Thus, although transport is catalysed by a population of different transporters, they behave as a single, saturable enzyme. From our derivation of the regulation coefficients (Section 1) it is apparent that changes of the K_m of glucose transport affect the metabolic regulation coefficient, rather than the hierarchical regulation coefficient. The metabolic regulation coefficient expresses the regulation through the *interaction* with the rest of metabolism. If there is a change in V_{max} without a change in K_m, it is classified as hierarchical regulation. If, through the expression of different isoenzymes, there is a change in effective K_m without a change in V_{max} this is classified as *metabolic* regulation, even though also in this case the mechanism by which the metabolic regulation is effected involves changes in gene expression.

More generally, isoenzymes with different kinetic properties that can be differentially expressed in response to environmental changes introduce the possibility of regulating both the quantity and quality of enzyme populations through hierarchical processes (e.g., gene expression, directed degradation of specific proteins, etc.). This feature of biological complexity is reflected in a more complicated interpretation of regulation analysis results, because gene-expression processes may influence both sides around the multiplication sign in Eq. (1), and there is no longer cross independence between the multipliers. However there is still cross independence between the capacity (V_{max}) of the enzyme and a function describing its interaction with the rest of metabolism $g([Glc]_{out}, [Glc]_{in}, K_{m,out}, K_{m,in}, \alpha)$, in the case of a symmetrical carrier (Eqs. (5) and (6)). Regulation analysis remains useful therefore, be it that more generally ρ_h describes the regulation of the flux through changes in enzyme capacity and ρ_m describes the regulation of the flux through changes in the interaction of the enzyme with the rest of metabolism (here regulation can be due to a change in metabolism, or e.g., through changes in K_m due to isoenzyme expression or due to stable phosphorylation of the enzyme).

Moreover, in the case in which there are two isoenzymes, of which the overall kinetics cannot be described by a single-component Michaelis–Menten equation, Eq. (1) becomes:

$$\begin{aligned} v &= v_1(e_1, \mathbf{X}) + v_2(e_2, \mathbf{X}) \\ &= f_1(e_1) \cdot g_1(\mathbf{X}) + f_2(e_2) \cdot g_2(\mathbf{X}). \end{aligned} \tag{7}$$

Equating the functions of the enzyme concentrations with the corresponding V_{max}'s this can be written as

$$v = V'_{max} \cdot g'(\mathbf{X}, V_{max\,1}/V_{max\,2}) \tag{8}$$

with:

$$V'_{max} = V_{max\,1} + V_{max\,2}, \tag{9}$$

$$\begin{aligned} g'\left(X, \frac{V_{max\,1}}{V_{max\,2}}\right) &= \frac{V_{max\,1}}{V_{max\,1} + V_{max\,2}} \cdot g_1(\mathbf{X}) \\ &\quad + \frac{V_{max\,2}}{V_{max\,1} + V_{max\,2}} \cdot g_2(\mathbf{X}), \end{aligned} \tag{10}$$

$$g'\left(X, \frac{V_{max\,1}}{V_{max\,2}}\right) = \frac{\frac{V_{max\,1}}{V_{max\,2}}}{\frac{V_{max\,1}}{V_{max\,2}} + 1} \cdot g_1(X) + \frac{1}{\frac{V_{max\,1}}{V_{max\,2}} + 1} \cdot g_2(X). \tag{11}$$

The usual regulation analysis can now be applied, where the hierarchical regulation coefficient retains its meaning as the only term expressing the dependence on total V_{max}, whilst the metabolic regulation coefficients now comprise not only the classical type of metabolic regulation, but also regulation through a possible shift in kinetic properties due to a shift in isoenzyme expression.

Until now we have only separated regulation by changes of V_{max} (catalytic capacity) from changes in metabolism. Changes in V_{max} are the outcome of regulation at various levels, including transcription, translation, mRNA and protein degradation, and post-translational modifications that affect the activity of enzymes. Currently we are developing an extension of regulation analysis that allows quantifying the relative contributions of these processes. Thus, regulation can be dissected quantitatively into its separate mechanisms. First, metabolic regulation should be dissected from hierarchical regulation, and only if hierarchical regulation is important, the different processes in the hierarchy need to be analyzed in full detail. In this light, we think that our study may be a first step towards a comprehensive analysis of regulation of cell function.

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