The fluxes through glycolytic enzymes in *Saccharomyces cerevisiae* are predominantly regulated at posttranscriptional levels

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Metabolic fluxes may be regulated "hierarchically," e.g., by changes of gene expression that adjust enzyme capacities (V_{max}) and/or "metabolically" by interactions of enzymes with substrates, products, or allosteric effectors. In the present study, a method is developed to dissect the hierarchical regulation into contributions by transcription, translation, protein degradation, and posttranslational modification. The method was applied to the regulation of fluxes through individual glycolytic enzymes when the yeast *Saccharomyces cerevisiae* was confronted with the absence of oxygen and the presence of benzoic acid depleting its ATP. Metabolic regulation largely contributed to the ≈10-fold change in flux through the glycolytic enzymes. This contribution varied from 50 to 80%, depending on the glycolytic step and the cultivation condition tested. Within the 50–20% hierarchical regulation of fluxes, transcription played a minor role, whereas regulation of protein synthesis or degradation was the most important. These also contributed to 75–100% of the regulation of protein levels.

gene-expression cascade | glycolysis | posttranscriptional regulation | regulation analysis | systems biology

The 1990s have witnessed a revolution in molecular cell biology. Nucleotide sequences of complete genomes were elucidated, and new techniques enabled genome-wide analysis of mRNA and protein concentrations and accurate estimates of metabolic flux distributions (1). The central dogma of molecular biology is that DNA encodes mRNA and mRNA encodes proteins, which in turn fulfill the many functions in the cell. Therefore, a strong correlation was anticipated among mRNA concentrations, protein concentrations, and metabolic fluxes. However, subsequent gene-expression studies led to the paradoxical conclusion that correlations between mRNA levels and protein levels (2, 3), between mRNA and *in vivo* fluxes (4, 5), and between enzyme activities and fluxes (6, 7) were far from perfect.

There are several explanations for the lack of correlation between the different levels of gene expression. Clearly defined and strictly controlled cultivation methods are required to obtain high-quality datasets (8, 9). Furthermore, there should be a time delay between changes at the mRNA level and the corresponding changes of protein concentrations and enzyme activities. However, even in steady-state chemostat cultures, in which the cells grow in a constant environment for prolonged periods of time, mRNA levels, protein concentrations/activities, and fluxes correlated poorly (4, 6, 10). A remaining explanation might be that much of the regulation of gene expression is posttranscriptional. Indeed, regulatory mechanisms that affect translation, protein degradation, posttranslational modification of proteins, and enzymes directly have been documented extensively. High-throughput measurements of translation rates and protein turnover in *Saccharomyces cerevisiae* showed that these varied significantly between proteins and conditions (11–13). Posttranslational modifications of proteins and metabolic regulation need to be considered as well (7, 14).

The question then becomes how one should quantify and integrate all these different levels of regulation to come to a coherent understanding of the regulation of cell function. This question is addressed by regulation analysis (6, 7, 14). In its original version, regulation analysis enables a quantitative dissection of the regulation of *in vivo* enzyme fluxes by gene expression on the one hand and metabolism on the other as follows:

$$\frac{\Delta \log f(e)}{\Delta \log J} + \frac{\Delta \log g(\mathbf{X},\mathbf{K})}{\Delta \log J} = \frac{\Delta \log V_{\max}}{\Delta \log J} + \frac{\Delta \log g(\mathbf{X},\mathbf{K})}{\Delta \log J} = \rho_h + \rho_m = 1, \quad [1]$$

where e is the concentration of the enzyme; $\mathbf{X}$ is a vector of concentrations of substrates, products, and other metabolic effectors; $\mathbf{K}$ is a vector of affinity constants parameterizing the strength with which the enzyme interacts with its substrates, products, and allosteric effectors; and J is the steady-state flux. The function $f(e)$ usually equals the maximum capacity (V_{max}) of the enzyme, and the function $g(\mathbf{X},\mathbf{K})$ describes its interaction with the rest of metabolism.

In Eq. **1**, ρ_h is the hierarchical regulation coefficient, which quantifies to what extent the local flux through the enzyme is regulated by a change in enzyme capacity (V_{max}). Such a change may be effected by the hierarchical cascade of gene expression, from transcription to posttranslational modification. ρ_m is the metabolic regulation coefficient, which quantifies the relative contribution of changes in the interaction of the enzyme with the rest of metabolism to the regulation of the enzyme's local flux. ρ_m includes regulation through changes in metabolite concentrations and changes in the affinity of the isoenzymes, e.g., through shifts in isoenzyme expression (14). In practice, the hierarchical regulation coefficient ρ_h is readily determined whenever the V_{max} and the flux through the enzyme J can be measured or estimated. The metabolic regulation coefficient ρ_m then follows from the summation theorem expressed in Eq. **1**.

However, as presented, this method does not enable the quantification of the contribution of transcription, mRNA degradation,

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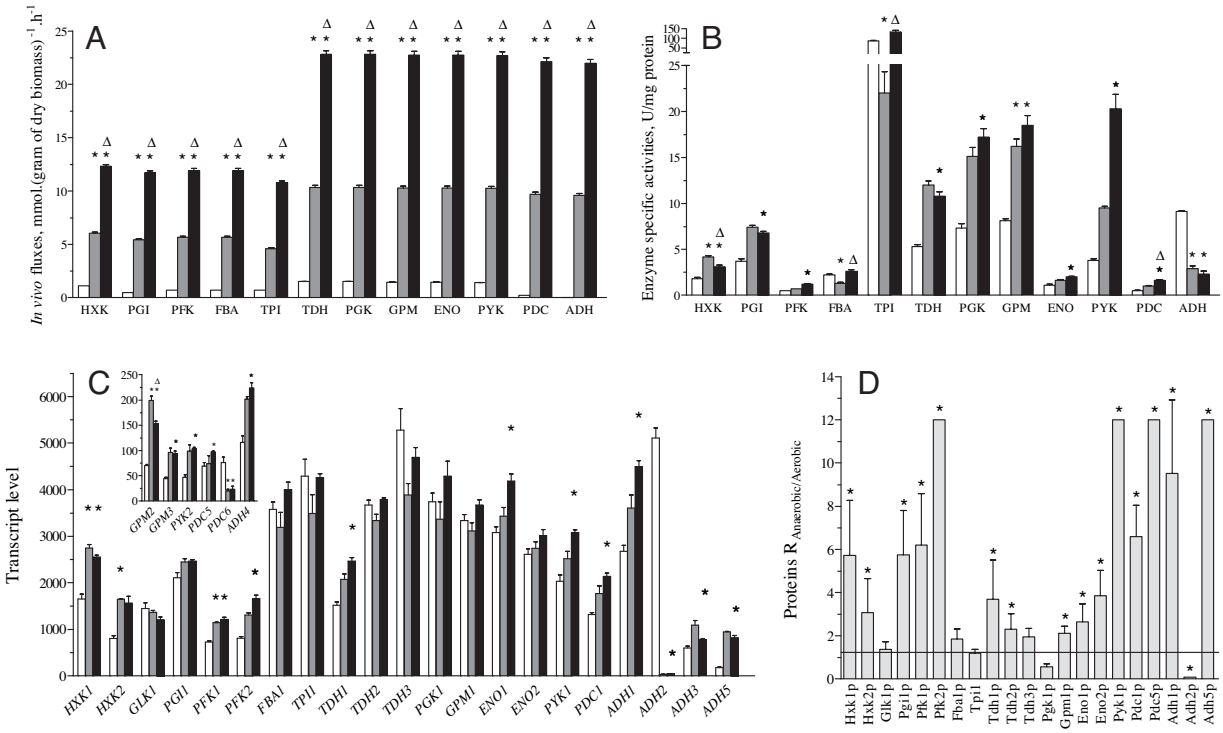

Fig. 1. Cellular levels and *in vivo* fluxes of *S. cerevisiae* glycolysis cultivated in glucose-limited chemostats in aerobiosis or anaerobiosis with or without benzoic acid. (*A*) *In vivo* fluxes, calculated from measured external metabolites. (*B*) Specific enzyme activities (V_{max}). (*C*) Transcript levels. (*D*) Protein levels of *S. cerevisiae* cultivated in glucose-limited anaerobic versus aerobic chemostats. White bars represent aerobic cultures, gray bars represent anaerobic cultures, and black bars represent anaerobic cultures with benzoate, except in *D*, where gray bars represent the ratio of anaerobic to aerobic expression. Asterisks indicate significant changes as compared with aerobic chemostats (Student's *t* test, $P = <0.01$). Triangles indicate significant changes between anaerobic cultures with and without benzoic acid (Student's *t* test, $P = <0.01$).

translation, protein degradation, or posttranslational modification to the regulation of V_{max}. The aim of the present study is to demonstrate that the hierarchical regulation can be dissected in terms of the relative contributions of these different regulatory mechanisms. This advanced regulation analysis is applied to glycolysis in the yeast *S. cerevisiae*. This is an abundant pathway in a well studied organism, and it undergoes large flux changes on perturbations. Yeast will be grown in steady-state chemostat cultures, first because this allows us to study long-term responses in which gene-expression regulation is likely to be more important than during initial responses, and second because chemostat studies are most suitable to collect the quantitative and reproducible data that are required (8). Altogether, it is a well suited model system for a quantitative dissection of complex regulation. Regulation of the expression of the glycolytic enzymes will be investigated in two important situations that result in drastic changes in the glycolytic fluxes. To quantify the regulation of the V_{max} values and the fluxes at the different levels of gene expression, we measured how the fluxes through the glycolytic enzymes, the V_{max} values, and the concentrations of these enzymes and their corresponding mRNA concentrations change when yeast is exposed to these challenges.

Results

Are Fluxes Regulated by the Hierarchical Cascade of Gene Expression or by Metabolic Interactions? In all steady-state glucose-limited chemostat experiments, *S. cerevisiae* was cultivated at the same dilution rate and thus specific growth rate (μ) of 0.10 h^{-1}. The three different cultivation conditions applied (aerobic, anaerobic, and anaerobic in the presence of benzoic acid) were chosen with the specific aim of covering a wide range of glycolytic fluxes. In aerobic glucose-limited chemostat cultures, glucose was dissimilated fully respiratorily into biomass and carbon dioxide, whereas in the anaerobic glucose-limited chemostat cultures at the same dilution rate, *S. cerevisiae* displayed a fully fermentative metabolism, producing ethanol, glycerol, carbon dioxide, and biomass formation [supporting information (SI) *Appendix*, section 1]. This of course implied drastic changes for the calculated *in vivo* fluxes through pyruvate decarboxylase and alcohol dehydrogenase, the two enzymes of alcoholic fermentation. Furthermore, the ATP yield from alcoholic fermentation is much lower than that from respiratory glucose dissimilation. To compensate for this lower yield, the carbon fluxes through the glycolytic enzymes were 5- to 11-fold higher in the anaerobic than in the aerobic cultures (Fig. 1*A*). To further increase the carbon fluxes in glycolysis, the nonmetabolizable weak acid benzoate was added. Weak acids cause an increased ATP requirement that is met by increased rates of glucose dissimilation (15). This was reflected in a 2-fold increase in the fluxes through the glycolytic enzymes when 2 mM benzoic acid was added to anaerobic cultures (Fig. 1*A*).

To define to what extent the observed flux changes were caused by changes in V_{max} via regulation of the hierarchical events leading from gene transcription to active enzyme, the contribution of the change in V_{max} to the change in flux through each enzyme (the so-called hierarchical regulation coefficient ρ_h) was determined by regulation analysis (Tables 1 and 2). A reaction that is purely regulated by the cascade of gene expression would have a ρ_h of 1. Conversely, a reaction that is solely metabolically regulated would have a ρ_h of 0 (see Eq. **1**). In most cases, the changes in fluxes resulted from both hierarchical and metabolic regulatory mechanisms, but their respective contribution clearly depended on the reaction considered and the culture conditions (Tables 1 and 2). When comparing aerobic and anaerobic cultures, we obtained ρ_h values between 0.2 and 0.5, meaning that hierarchical regulation was involved but was responsible for at most 50% (Table 1). The

Table 1. Regulation of glycolytic fluxes and V_{max} values in yeast in anaerobic compared to aerobic glucose-limited chemostats

	Hierarchical (ρ_h) and metabolic (ρ_m) regulation of flux			Transcription regulation of V_{max}		Transcription regulation of flux		Posttranslation regulation of V_{max}	
Reaction	ρ_h	SEM	ρ_m	$\rho_{mRNA,V_{max}}$	SEM	$\rho_{mRNA,flux}$	SEM	$\rho_{PT,V_{max}}$	SEM
HXK	0.50	0.06	0.50	0.45	0.07	0.23	0.02		
PGI	0.28	0.03	0.72	0.22	0.11	0.06	0.03	−1.51	0.28
PFK	0.15	0.02	0.85	1.48	0.22	0.22	0.02		
FBA	−0.24	0.05	1.24	0.25	0.28	−0.06	0.07	2.20	0.73
TPI	−0.72	0.07	1.72	0.16	0.13	−0.11	0.10	1.13	0.63
TDH	0.42	0.03	0.58	−0.14	0.11	−0.06	0.05	0.18	0.04
PGK	0.38	0.05	0.62	−0.16	0.21	−0.06	0.08	1.78	0.52
PGM	0.35	0.03	0.65	−0.02	0.11	−0.01	0.04	−0.08	0.01
ENO	0.23	0.06	0.77	0.18	0.15	0.04	0.03	−1.18	0.41
PYK	0.45	0.03	0.55	0.25	0.12	0.11	0.05		
PDC	0.19	0.02	0.81	0.31	0.16	0.06	0.03	−1.55	0.21
ADH				0.34	0.05				

remaining 50–80% of the flux changes was caused by metabolic regulation (Table 1, third column). In response to the presence of benzoic acid, causing an increased utilization of ATP, most of the fluxes through the glycolytic reactions doubled, whereas most V_{max} values hardly changed (Fig. 1 *A* and *B*). This resulted in small ρ_h values and ρ_m values close to 1 for most enzymes, indicating that these enzymes were predominantly regulated by metabolism (Table 2). There were a few notable exceptions. The V_{max} of phosphofructokinase, fructose-bisphosphate aldolase, triose-phosphate isomerase, and pyruvate kinase increased strongly, resulting in large hierarchical regulation coefficients for these enzymes.

Dissecting Hierarchical Regulation into Contributions by Transcription, Translation, Protein Degradation, and Posttranslational Modification: The Method. For those enzymes with a high ρ_h, i.e., the enzymes that were to a large extent regulated hierarchically, we were interested in quantifying the contributions of the various processes in the gene-expression cascade to that regulation. To analyze hierarchical regulation of V_{max} in more detail, the theory was extended as follows.

The measured V_{max} of an enzyme depends on its concentration and its turnover number k_{cat}:

$$V_{max} = k_{cat} \cdot [\text{protein}]. \qquad [2]$$

Table 2. Regulation of glycolytic V_{max} values and fluxes in anaerobic glucose-limited chemostats with versus without benzoic acid

	Hierarchical (ρ_h) and metabolic (ρ_m) regulation of flux			Transcription regulation of V_{max}		Transcription regulation of flux	
Reaction	ρ_h	SEM	ρ_m	$\rho_{mRNA,V_{max}}$	SEM	$\rho_{mRNA,flux}$	SEM
HXK	−0.43	0.09	1.43	0.26	0.16	−0.11	0.06
PGI	−0.11	0.05	1.11	−0.12	0.48	0.01	0.05
PFK	0.74	0.08	0.26	0.29	0.06	0.21	0.04
FBA	0.93	0.15	0.07	0.35	0.20	0.32	0.18
TPI	2.12	0.16	−1.12	0.12	0.08	0.26	0.18
TDH	−0.13	0.07	1.13	−1.59	1.05	0.21	0.08
PGK	0.17	0.11	0.83	1.94	1.73	0.32	0.20
PGM	0.17	0.10	0.83	1.07	0.78	0.18	0.08
ENO	0.27	0.08	0.73	0.73	0.34	0.20	0.07
PYK	0.95	0.11	0.05	0.27	0.10	0.26	0.09
PDC	0.54	0.09	0.46	0.46	0.26	0.25	0.13
ADH	−0.28	0.22	1.28	−0.36	0.36	0.10	0.06

Because the cultures under study were at steady state, the rate of translation v_{transl} should equal the rate of protein degradation plus the dilution of proteins due to cell growth, for

$$\frac{\mathrm{d}[\text{protein}]}{\mathrm{d}t} = \nu_{transl} - \nu_{degrad} - \nu_{dil} = 0. \qquad [3]$$

The rate of synthesis of any specific protein i ($v_{transl,i}$) was approximated by:

$$\nu_{transl,i} = k_{transl,i} \cdot [\text{ribosome}] \cdot [\text{mRNA}_i]. \qquad [4]$$

$k_{transl,i}$ represents a function of various variables, including the GTP/GDP ratio and the concentrations of aminoacyl tRNAs, but it should be independent of the concentration of ribosomes and the concentrations of the mRNAs encoding glycolytic proteins. The rates of dilution and degradation of each protein i were taken proportional to the concentration of that protein with proportionality constants μ and $k_{degrad,i}$, respectively, i.e.:

$$\frac{\mathrm{d}[\text{protein}_i]}{dt} = k_{transl,i} \cdot [\text{ribosome}] \cdot [\text{mRNA}_i] - k_{degrad,i} \cdot [\text{protein}] - \mu \cdot [\text{protein}_i] = 0. \qquad [5]$$

Combining Eq. **5** with Eq. **2** yields:

$$V_{max,i} = \frac{k_{cat,i} \cdot k_{transl.i} \cdot [\text{ribosome}] \cdot [\text{mRNA}_i]}{(\mu + k_{degrad})} \qquad [6]$$

The contribution of each of the components in Eq. **6** to an overall change of V_{max} of the enzyme was determined by taking the logarithm, calculating the difference between two conditions, and dividing by $\Delta\log V_{max}$:

$$\frac{\Delta\log V_{max,i}}{\Delta\log V_{max,i}} = \underbrace{\frac{\Delta\log k_{cat,i}}{\Delta\log V_{max,i}}}_{\text{posttranslational modifications}} + \underbrace{\left(\frac{\Delta\log k_{transl,i}}{\Delta\log V_{max,i}} + \frac{\Delta\log[\text{ribosome}]}{\Delta\log V_{max,i}}\right)}_{\text{translation}} + \underbrace{\frac{\Delta\log[\text{mRNA}_i]}{\Delta\log V_{max,i}}}_{\text{transcription}} - \underbrace{\frac{\Delta\log(\mu + k_{degrad,i})}{\Delta\log V_{max,i}}}_{\text{protein degration/growth}}$$

$$\equiv \rho_{i,PT,V_{max}} + \rho_{i,transl,V_{max}} + \rho_{i,mRNA,V_{max}} + \rho_{i,dil/degrad,V_{max}} = 1, \quad [7]$$

in which $\rho_{PT,V_{max}}$ quantifies the regulation of V_{max} by posttranslational modifications, $\rho_{transl,V_{max}}$ quantifies the regulation of V_{max} by translation activity, $\rho_{mRNA,V_{max}}$ quantifies the regulation of V_{max} by the mRNA concentration, and $\rho_{dil/degrad,V_{max}}$ quantifies the regulation of V_{max} by protein degradation and dilution due to growth. The sum of these four coefficients must be 1. The hierarchical regulation of the flux through each glycolytic enzyme can thus be dissected. For simplicity, the effect of the apparent rate constant of translation and the ribosome concentration were here grouped in $\rho_{transl,V_{max}}$. In principle, however, they can be measured separately. The only processes that could not be separated in this way were protein degradation and dilution due to growth. Because the specific growth rate is measured easily, it can be incorporated explicitly in the determination of $\rho_{degrad/dil,V_{max}}$.

Regulation of Fluxes and V_{max} by Transcript Levels. To quantify the importance of transcriptional regulation within the gene-expression cascade ($\rho_{i,mRNA,V_{max}}$), the mRNA levels of the 27 genes encoding the isoenzymes of glycolysis and fermentation were measured by microarray analysis (Fig. 1*C*). In anaerobic cultures, 16 of the 27 genes were significantly up-regulated, and two were down-regulated, compared with aerobic cultures. However, for most reactions, the increase in mRNA concentrations was smaller than the increase in *in vivo* V_{max} values. We focused on those enzymes that displayed a substantial change in V_{max} (ρ_h of 0.3 or higher). Among these enzymes, only hexokinase displayed a substantial $\rho_{mRNA,V_{max}}$ when comparing aerobic and anaerobic conditions, indicating that transcription was for 45% regulating its V_{max} (Table 1). To evaluate the overall regulation of the fluxes by mRNA levels, we calculated $\rho_{mRNA,flux}$, defined by:

$$\rho_{mRNA,flux} \equiv \frac{\Delta \log[mRNA]}{\Delta \log flux} = \rho_h \cdot \rho_{mRNA,V_{max}}. \quad [8]$$

In the comparison of aerobic and anaerobic cultures, $\rho_{mRNA,flux}$ was at most 0.2 (hexokinase and phosphofructokinase; Table 1, column 6), implying that the flux was regulated for 20% by the mRNA level in these cases. In all other cases, V_{max} values and fluxes were hardly regulated by mRNA levels as reflected by low mRNA regulation coefficients (Table 1).

When comparing anaerobic conditions with and without benzoic acid, the changes in V_{max} and in mRNA abundances were much smaller than between aerobicity and anaerobicity without benzoate (Fig. 1 *B* and *C*), which resulted in relatively high errors in the $\rho_{mRNA,V_{max}}$ (Table 2). The coefficients with reasonably good standard errors of the mean were low, with the exception of enolase and pyruvate decarboxylase (0.73 and 0.46, respectively). A number of $\rho_{mRNA,V_{max}}$ values were negative, but they were all small and not or hardly significantly different from zero.

To check whether the relatively small changes in transcription did not result from a lack of accuracy of the microarrays, the transcript levels of 11 glycolytic genes were measured by quantitative RT-PCR (*SI Appendix*, section 2). The tested genes displayed very similar changes in expression when using both techniques and thereby confirmed the conclusion that regulation by transcript levels was of minor importance.

In conclusion, the poor contribution of transcription to the regulation of V_{max} revealed that the regulation within the gene-expression cascade was exerted further downstream.

Regulation of Protein Levels by Translation and/or Protein Degradation. Although mRNA levels hardly regulated the overall fluxes and V_{max} of most glycolytic enzymes, this does not exclude that specific isoenzyme concentrations are regulated by changes at the mRNA level. To examine this scenario, concentrations of the glycolytic proteins were measured by liquid chromatography-MS/MS. By comparing proteins from *in vivo* ^{15}N- and ^{14}N-labeled aerobic and anaerobic chemostats, we could reliably identify and quantify 21 of the 27 glycolytic proteins (Fig. 1*D*). Five proteins (Pfk2p, Pyk1p, Pdc5p, Adh5p, and Adh2p) were identified in only one of the conditions and could not be considered for regulation analysis. Most proteins were significantly up-regulated under anaerobic conditions compared with aerobic conditions with the exceptions of Glk1p, Fba1p, Tpi1p, and Pgk1p, the expression of which hardly changed. The regulation of the level of a protein *i* by the corresponding mRNA level was quantified as follows:

$$\rho_{i,mRNA,protein} = \frac{\Delta \log[mRNA_i]}{\Delta \log[protein_i]}. \quad [9]$$

For the large majority of the proteins, the contribution of the change in corresponding mRNA level to the regulation of their concentration was <30% (Table 3, $\rho_{mRNA,protein} < 0.3$). This included proteins displaying 6-fold changes in concentration between aerobic and anaerobic chemostats (i.e., Pgi1p, Pfk1p, and Pdc1p). Oxygen-responsive proteins that were only detected under anaerobic conditions (Pfk2p, Pyk1p, and Pdc5) and could therefore not be used for regulation analysis were also clearly not regulated by transcription: Under aerobic conditions, their mRNAs were present and almost as abundant as under anaerobic conditions, whereas the corresponding proteins could not be detected under aerobic conditions (cf. Fig. 1 *C* and *D*). The only two proteins that were regulated substantially by their mRNA levels were Hxk2p ($\rho_{mRNA,protein}$ 0.65) and Adh2p. Of the latter, neither protein nor mRNA could be detected under anaerobic conditions.

Because the glycolytic protein concentrations were hardly regulated by mRNA levels, the ones that did change must have been regulated at the levels of translation and/or protein degradation. Revisiting Eqs. **3** and **4**, one finds:

$$[protein_i] = k_{transl,i} \cdot [ribosome] \cdot [mRNA_i]/(k_{degrad,i} + \mu) \quad [10]$$

and therefore:

Table 3. Transcription and translation/degradation regulation of the concentrations of glycolytic enzymes in anaerobic versus aerobic glucose-limited chemostats

	Transcription regulation of protein concentration		Translation and degradation regulation of protein concentration
Enzyme	$\rho_{mRNA,protein}$	SEM	$\rho_{i,transl,protein} + \rho_{i,degrad,protein}$
Hxk1p	0.30	0.10	0.70
Hxk2p	0.65	0.17	0.35
Glk1p	−0.14	0.45	1.14
Pgi1p	0.09	0.05	0.91
Pfk1p	0.25	0.07	0.75
Fba1p	−0.20	0.27	1.20
Tpi1p	−1.25	7.19	2.25
Tdh1p	0.24	0.09	0.76
Tdh2p	−0.12	0.08	1.12
Tdh3p	−0.44	0.42	1.44
Pgk1p	0.21	0.52	0.79
Gpm1p	−0.09	0.13	1.09
Eno1p	0.11	0.09	0.89
Eno2p	0.04	0.06	0.96
Pdc1p	0.15	0.06	0.85
Adh1p	0.13	0.07	0.87

$$\frac{\Delta \log[\text{protein}_i]}{\Delta \log[\text{protein}_i]} = \underbrace{\left(\frac{\Delta \log k_{\text{transl},i}}{\Delta \log[\text{protein}_i]} + \frac{\Delta \log[\text{ribosome}]}{\Delta \log[\text{protein}_i]} \right)}_{\text{translation}} + \underbrace{\frac{\Delta \log[\text{mRNA}_i]}{\Delta \log[\text{protein}_i]}}_{\text{transcription}} - \underbrace{\frac{\Delta \log(\mu + k_{\text{degrad},i})}{\Delta \log[\text{protein}_i]}}_{\text{protein degration/growth}}$$

$$\equiv \rho_{i,\text{transl,protein}} + \rho_{i,\text{mRNA,protein}} + \rho_{\text{i,dil/degrad,protein}} = 1. \quad [11]$$

Because the specific growth rate and therefore the dilution of proteins are constant in the present study, it follows that the regulation of the concentration of any protein through translation and degradation together ($\rho_{i,\text{transl,protein}} + \rho_{i,\text{degrad,protein}}$) can be calculated by subtracting the regulation through mRNA concentrations from 1. The final column of Table 3 gives the results of this calculation and shows that the regulation of the concentration of the glycolytic proteins in yeast appears to be regulated by translation or protein degradation much more than by transcription.

Posttranslational Regulation. We showed that the hierarchical regulation of $V_{\max}$ of the glycolytic enzymes did not occur at the transcription level and therefore must have occurred posttranscriptionally. Changes in $V_{\max}$ could result from (*i*) tuning of protein concentration by translation and/or degradation or (*ii*) modification of the kinetic properties of the enzymes by posttranslational modification. We have also shown that the protein concentrations under study were regulated largely by protein translation and/or degradation. To investigate the contribution of posttranslational processes to the regulation of $V_{\max}$, $\rho_{PT,V_{\max}}$ was calculated (cf. Eq. **7**).

For most of the resulting regulation coefficients for posttranslational modification $\rho_{PT,V_{\max}}$, the standard deviation of the mean was unfortunately too large to conclude anything about the occurrence of protein modifications. However, two enzymes with relatively low standard deviations (glyceraldehyde-3-phosphate dehydrogenase and phosphoglycerate mutase) were only marginally regulated by posttranslational processes.

Discussion

In this study, we developed the theory to quantitatively dissect the regulation of gene expression into contributions by each of the processes in the gene-expression cascade. Using this theory, we investigated the regulatory events responsible for the tuning of the capacity and activity of the glycolytic enzymes in bakers' yeast under three growth conditions under which the local glycolytic fluxes covered a complete order of magnitude. Our main conclusion is that, to the extent that the fluxes through glycolytic enzymes in *S. cerevisiae* are regulated by gene expression at all, regulation by mRNA levels plays a marginal role. Rather, most of the observed gene-expression regulation was exerted at the levels of protein synthesis and/or degradation and possibly also at the level of posttranslational modification of enzymes. To our knowledge, this is the first time that the regulation of a complete metabolic pathway has been dissected into contributions of the various processes in the gene-expression cascade. The results put the importance that many biologists attach to transcriptome analysis as a measure of gene expression in a new perspective.

First, we determined the quantitative importance of gene-expression regulation of the glycolytic flux upon removal of oxygen and addition of benzoic acid. In accordance with the results that were obtained for trypanosomes (6) and starving yeast cells (7), the whole spectrum of regulation strategies was observed, including purely hierarchical regulation (ρ_h close to 1), purely metabolic regulation (ρ_m close to 1), cooperative regulation (both ρ_h and ρ_m between 0 and 1), and antagonistic regulation (either ρ_h or ρ_m negative). The new results substantiate the earlier conclusion (7) that simple strategies of regulation, like multisite modulation (all enzymes purely and equally hierarchical), single enzyme regulation (one enzyme purely hierarchical and the others metabolic), or purely metabolic regulation (no hierarchical regulation at all) are not the rule. This is by no means trivial because, for example, multisite modulation would be very effective to achieve metabolite homeostasis (16). In reality, however, regulation was the result of a more complex mixture of gene expression and metabolic effects, which may reflect that the cell needs to optimize a number of different variables (fluxes, metabolite concentrations, protein concentrations) simultaneously, and there is no single optimization criterion like metabolite homeostasis or protein economy. In line with previous studies (6, 7) we observed that: (*i*) metabolic regulation was a substantial component of almost all regulation observed; (*ii*) different enzymes in the pathway tended to be regulated differently; (*iii*) in many cases, there was both metabolic and gene-expression regulation but to different extents; (*iv*) for some enzymes in some conditions, metabolic and gene-expression regulation were antagonistic; and (*v*) the same enzyme was often regulated differently in response to different challenges.

The principle of regulation analysis was then broadened to integrate new cellular levels and to discriminate between the various regulatory processes involved in the hierarchical (or gene expression) regulation. In the specific elaboration of this analysis method, we assumed that the protein concentrations were at steady state, a condition that is met in steady-state chemostats. Because the amount of any particular protein is low compared with the sum of the amounts of all protein species in the cell, we considered it likely that the rate of degradation of a protein was first order with respect to its concentration. Protein turnover measurements in chemostat cultures of *S. cerevisiae* support this hypothesis (12). In fact, this assumption is not required if protein degradation is negligible compared with dilution of proteins due to growth (Eqs. **5**–**7**). In the present steady-state analysis of growing cells, the latter seems realistic. Furthermore, we assumed the rate of translation to depend proportionally on the concentration of the mRNA of interest (Eq. **4**). This may seem to be a very strong assumption because ribosomes are catalysts that could become saturated. A single type of mRNA, however, has to compete with a large pool of other mRNA molecules. This decreases the apparent affinity of the mRNA for the ribosome, which should lead to a proportional dependence even if the ribosomes are saturated with total mRNA. To then calculate the regulation of $V_{\max}$ by the mRNA concentration, the expression levels of mRNAs encoding isoenzymes were summed. This is a simplification because isoenzymes often have different kinetic characteristics, and translation yield may well differ between mRNAs encoding isoenzymes. In the present study, this simplification was necessary; however, in future, more detailed studies, it could be avoided by explicitly taking into account the catalytic turnover numbers (k_{cat}) of the isoenzymes. Another such simplification was the description of the activity of each step in the glycolytic pathway in terms of a single $V_{\max}$. In reality, each isoenzyme has its own $V_{\max}$, and here a higher-resolution analysis should also be useful.

The attempt to identify the potential regulation of $V_{\max}$ by posttranslational processes highlighted the requirement of highly accurate data. Protein quantification based on 2D gel analysis resulted in standard errors exceeding the regulation coefficients themselves (data not shown). The protein expression ratios generated by the nano-liquid chromatography/MS-MS approach were more accurate (Fig. 1*D*). Yet, among the 12 coefficients assessing the contribution of posttranslational processes to the regulation of enzyme capacities, only two could be estimated reliably (glyceraldehyde-3-phosphate dehydrogenase and phosphoglycerate mutase), and they were small, implying that in these cases posttranslational modifications contributed little to $V_{\max}$ regulation. So far, potential posttranslational modifications have not been investigated systematically for all glycolytic enzymes. Among the few reports,

phosphorylation seems to be the predominant mechanism for protein modification. A few proteins have been demonstrated to be phosphorylated *in vitro* and/or *in vivo* [Hxk1 and Hxk2, Pyk1 and Pyk2, Eno1 and Eno2, and Gpm1p (17–19)], but the impact of phosphorylation on the activity has not been assessed in all cases. Unfortunately, our data were insufficient to estimate the regulation of hexokinase and pyruvate kinase by posttranslational modification. Enolase was hardly regulated by gene expression at all ($\rho_h < 0.3$), whereas phosphoglycerate mutase was not regulated by posttranslational modification (Table 1).

Our data were accurate enough to firmly establish that the regulation of glycolytic protein concentrations in yeast was mainly at the level of translation and degradation and hardly at the mRNA level (Table 3). Little is known about the mechanisms of translational regulation of the glycolytic and fermentative proteins. Of the few reports of translational regulation of specific protein, most address transcription factors (Gcn4p, Yap1p, and Yap2p) (20, 21). To date, in *S. cerevisiae*, Cpa1p is the only enzyme experimentally shown to be regulated translationally (22). All of the mRNAs encoding these proteins contain upstream open-reading frames that can partly or completely disable the initiation of translation (23). However, how the translation of the glycolytic proteins is regulated is unknown, and all of the mechanisms that have been described in yeast, such as the presence of upstream ORFs or mRNA binding proteins, should be considered (23). Even less is known about degradation of glycolytic enzymes. Although targeted degradation of enolase 2 has already been reported (24), regulatory mechanisms involving targeted degradation of glycolytic enzymes have not been systematically investigated. However, recent studies demonstrated that translation rates can be measured at the genome-scale, using microarrays, and suggested that regulation of translation rate to adjust protein concentration is a mechanism more widely spread in yeast that anticipated (11, 13). Our results urge further studies in which translation and degradation rates of individual proteins will be measured directly to quantitate their regulation more precisely.

For decades, attempts have been made to increase the fermentative capacity (i.e., the glycolytic rate) of bakers' yeast via genetic engineering (25, 26). So far, all of these attempts have failed. The present in-depth analysis of the glycolytic pathway reveals a complex and intricate regulation of the glycolytic flux. Regulation of glycolysis is not only exerted by expression of the glycolytic genes but resides to a large extent in the interactions of the glycolytic proteins with their environment. The latter observation may, at least partly, clarify the past failures of genetic engineering through manipulation of gene expression and suggests that metabolic engineers face a major challenge to further enhance fermentative capacity in bakers' yeast.

More generally, the demonstration that such a central process as yeast glycolysis is regulated much less by transcription than perhaps anticipated and in fact through many regulatory mechanisms at the same time suggests that this might also be the case for other pathways, organisms, and conditions. Perhaps identification of where the more important regulatory mechanisms are deserves to be prioritized compared with an immediate or exclusive focus on transcriptomics or proteomics. This then paves the way for more in-depth studies in which regulation will be dissected quantitatively as a function of time.

Materials and Methods

Strain and Growth Conditions. The *S. cerevisiae* strain CEN.PK113–7D (*MAT*a, *MAL2–8*^{c}, *SUC2*) was grown at 30°C in 2-liter fermenters (Applikon, Schiedam, The Netherlands) as described in refs. 15 and 27. For the anaerobic chemostats with benzoic acid, 2 mM sodium benzoate was added to the feed medium.

Analytical Methods. Culture supernatants and media were analyzed by HPLC. Culture dry weights were determined as in ref. 28.

Microarray Analysis. Affymetrix (Santa Clara, CA) microarrays were performed as described in ref. 8. The results were derived from five independent aerobic cultures, four anaerobic cultures, and three anaerobic cultures with benzoic acid. Acquisition and quantification of array images were performed by using the Affymetrix MAS software, Version 5.0.

Protein Analysis by Liquid Chromatography/MS-MS. The detailed approach for protein analysis using *in vivo* ^{15}N protein labeling can be found in ref. 29.

Enzyme Assays. Activity of glycolytic enzymes in cell extracts was assayed according to ref. 30. The enzyme activities presented in this work are the average of measurements in samples from at least three independent culture replicates.

Metabolic Flux Distribution. Intracellular metabolic fluxes in growing cells were calculated through metabolic flux balancing as described in ref. 4.

Regulation Analysis. A detailed description of ρ_h, ρ_m, and a standard error calculation can be found in *SI Appendix*, section 4.

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