Unraveling the complexity of flux regulation

new insights through the quantitative description of regulatory processes

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Abbreviations and symbols

2PG	2-phosphoglycerate
3PG	3-phosphoglycerate
AA	aminoacids synthesis pathways
ACE	acetaldehyde
ADH	alcohol dehydrogenase –E.C. 1.1.1.1
ALD	fluctose bisphosphate aldolase –E.C. 4.1.2.13
BPG	1,3-bisphosphoglycerate
$C^J_{e_i}$	flux control coefficient of enzyme i
$C^{x_j}_{e_i}$	concentration control coefficient of enzyme i on metabolite x_j
DHAP	dihydroxyacetonephosphate
$\varepsilon^{v_i}_{x_j}$	elasticity of the rate v_i for the metabolite x_j
ENO	enolase –E.C. 2.7.1.40
EtOH	ethanol
F6P	fructose-6-phosphate
F16P	fructose-1,6-bisphosphate
Γ	mass-actio ratio
G6P	glucose-6-phosphate
GAP	glyceraldehyde-3-phosphate
GAPDH	glyceraldehyde-3-phosphate dehydrogenase –E.C.1.2.1.12
GLC	glucose
GLCi	intracellular glucose
GLT	glucose transporter
HK	hexokinase –E.C. 2.7.1.1
J	steady-state flux
k_{cat}	catalytic constant of an enzyme
k_{deg}	first order kinetic constant of the rate of protein degradation
K_{Eq}	equilibrium constant
K_m	Michaelis-Menten constant

k_{trans}	first order kinetic constant of the rate of translation
P	reaction of pathway product
${}^{v}P_{x_i}^{J}$	partitioned response coefficient for the metabolite x_i
PEP	phosphoenolpyruvate
PDC	pyruvate decarboxylase –E.C. 4.1.1.1
PFK	6-phosphofructokinase –E.C. 2.7.1.11
PGI	glucose-6-phosphate isomerase –E.C. 5.3.1.9
PGK	phosphoglycerate kinase –E.C. 2.7.2.3
PGM	phosphoglycerate mutase –E.C. 5.4.2.1
PK	pyruvate kinase –E.C. 2.7.1.40
PKA	protein kinase A –E.C. 2.7.11.11
PPP	pentose phosphate pathway
PYR	pyruvate
$\rho_{dd,protein}$	degradation/dilution regulation coefficient for a protein
$\rho_{dd,V_{max}}$	degradation/dilution regulation coefficient for V_{max}
ρ_h	hierarchical regulation coefficient
ρ_m	metabolic regualtion coefficient
$\rho_{mRNA,flux}$	transcriptional regulation coefficient for an enzyme flux
$\rho_{mRNA,protein}$	transcriptional regulation coefficient for a protein
$\rho_{mRNA,V_{max}}$	transcriptional regulation coefficient for V_{max}
$\rho_{PT,Vmax}$	posttranslational regulation coefficient for V_{max}
$\rho_{trans,V_{max}}$	translational regulation coeffcient for V_{max}
$\rho_{trans,protein}$	translational regulation coefficient for a protein
R_i^j	response coefficent of the variable or parameter i on the variable j
S	reaction or pathway substrate
SD	standard deviation
SEM	standard error of the mean
SC	storage carbohydrates –glycogen and trehalose
TPI	triose-phosphate isomerase –E.C. 5.3.1.1.
v	enzyme rate
v_{trans}	rate of translation
v_{deg}	rate of protein degradation
v_{dil}	rate of protein dilution due to growth

Summary

This thesis is concerned with the study of the regulatory processes involved in the adaptation of metabolic systems to environmental and genetic changes. The study of regulation is an endeavor unique to biology. It addresses systems of a complexity that is unparalleled in the inanimate realm. More importantly, these systems are adaptive: living cells modulate their system properties in response to environmental changes. These modulations are governed by yet unknown drives and constraints.

In the introductory chapter (Chapter 1) it is argued that our understanding of regulatory processes is hindered by the lack of a precise definition of the term regulation and of appropriate methodologies to describe regulatory process in an unambiguous and quantitative manner. Chapters 2 to 5 report the implementation, evaluation and further development of Regulation Analysis, a method that enables the quantitative description of the regulation of enzyme rates and their catalytic capacities. This method was implemented, tested and elaborated in a series of investigations upon the regulation of *Saccharomyces cerevisiae*'s glycolysis to nutrient starvation, oxygen deprivation, increased free-energy dissipation by addition of benzoic acid, or deletion of the gene *HXK2* encoding hexokinase II. The experimental findings and analyses reported in this thesis yielded new insights into the complexity of the regulation of metabolic fluxes and the catalytic capacities of the enzymes catalyzing their reactions.

In the past, several efforts have been made to devise a quantitative framework for the study of metabolic regulation. Of these, Regulation Analysis stands out as a method suitable for the *experimental* study of regulatory processes. Regulation Analysis quantitatively dissects the contributions of changes in enzyme capacities (V_{max} –called hierarchical regulation) and changes in the way enzymes interact with the rest of metabolism (called metabolic regulation) to the local regulation of enzyme rates. This dissection is based on a property of most enzyme-catalyzed reactions: the rate of catalysis is *directly* proportional to the amount of active enzyme. Regulation Analysis introduces the possibility of making unambiguous and quantitative descriptions of the regulation of fluxes through individual enzymes embedded in biochemical networks of any complexity, in response to any number or kind of simultaneous perturbations.

Regulation Analysis is used throughout this thesis to describe the regulation of fluxes through individual glycolytic and fermentative enzymes in the yeast *Saccharomyces cerevisiae* when it adapts to a variety of environmental and genetic changes. Chapter 2 gives a detailed description of the method and introduces precise biochemical interpretations for all possible numerical outcomes of the analysis. Further, it refines the original interpretation of both hierarchical and metabolic regulation so

as to accommodate the common feature of differential expression of isoenzymes with different kinetic properties.

In Chapter 3, Regulation Analysis is used to formulate testable predictions of three hypotheses on the global regulation of pathway fluxes in terms of the local regulation of the enzyme rates in that pathway. These hypotheses were: exclusive metabolic regulation, single-site modulation and multi-site modulation. The first hypothesis predicts that all enzyme rates are regulated metabolically (*i.e.* the absence of hierarchical regulation); the second hypothesis, predicts that a single "key" metabolic step is regulated hierarchically while all other steps are regulated metabolically. The third hypothesis, predicts exclusive hierarchical regulation of all the enzymes rates in the pathway so as to ensure the homeostasis of metabolite concentrations. Chapters 3 , 4 and 5 describe the regulation of fluxes through individual glycolytic and fermentative enzymes when cultures of *Saccharomyces cerevisiae* adapted to: nitrogen or carbon starvation, oxygen deprivation, increased free-energy dissipation by addition of benzoic acid and the deletion of the gene *HXK2* encoding hexokinase II. These studies taken together allow the following conclusions: (i) metabolic regulation is often an important contributor to the local regulation of enzyme rates, (ii) living yeast cells use all possible combinations of hierarchical and metabolic regulation to modulate the rates of individual enzymes, (iii) fluxes through enzymes in a common pathway are regulated in different ways, suggesting that they play different regulatory roles in the regulation of the pathway's flux, (iv) the same metabolic step is often regulated differently when cells adapt to different perturbations, (v) the suggested hypotheses on the global regulation of metabolic pathway fluxes were falsified for the conditions tested, implying that they are not general, and (vi) the regulation of glycolytic and fermentative fluxes is often regulated by changes within as well as without the pathway's enzymes. These findings suggest that pathway fluxes are regulated in a subtle way with different enzymes playing different regulatory roles and show that the regulation of pathway fluxes need not to be governed by single drives or constraints. They also urge the formulation of new hypotheses on the global regulation of pathway fluxes.

An extension of the scope of Regulation Analysis to quantify the regulation of enzyme amounts and catalytic capacities in terms of the contributions of changes in mRNA concentration, translation and protein degradation rates, and posttranslational modifications is developed in Chapter 4. The analysis is based on the assumptions that protein concentrations are at steady-state and that the rates of translation and degradation of individual protein species are directly proportional to the corresponding concentrations of mRNA and protein. While the former assumption is likely to be warranted by the use of chemostat cultures, the latter two still require experimental verification, which will require increased precision of the available analytical techniques. The assumptions of direct proportionality of translation and protein degradation rates with respect to the concentrations of the corresponding mRNA and protein are based on the expectation that the machineries involved (ribosomes and proteasome) are unspecific and that the concentration of any single mRNA or protein species represents a minority in the population of all other mRNA or protein species.

In Chapter 4 this extended Regulation Analysis is applied to study the regulation of glycolytic enzyme amounts and capacities when *S. cerevisiae* adapts to anaerobiosis or to the presence of benzoic acid. Experiments showed that mRNA concentration

changes correlate poorly with the changes of enzyme amounts and capacities. The analysis suggests that mRNA changes account for less than 50% of the regulation of the glycolytic enzyme amounts and capacities and that changes in the rates of translation and/or protein degradation are the main regulators of protein amounts. Attempts to quantify the contributions of posttranslational modifications to the regulation of enzyme capacities highlighted the need for more accurate and reproducible proteomics. Although the standard errors of the mean were too large to be decisive, the occurrence of posttranslational modifications affecting the catalytic capacities of triosephosphate isomerase and phosphoglycerate kinase is suggested.

The methodologies used and developed in this thesis provide a quantitative framework with which experimental testing of hypotheses on the drives and constraints governing regulatory processes is made possible. Application of these methodologies to describe real regulatory processes in living cells has provided insights into a previously undescribed complexity of metabolic regulatory processes. It has shown that processes that have received relatively little attention such as metabolic regulation of fluxes, translation and protein degradation rates, are likely to play a major role in the regulation of metabolic systems.

Contents

Chapter 1

General Introduction

This thesis is concerned with the study of regulatory processes involved in the adaptation of glycolysis in the bakers' yeast *Saccharomyces cerevisiae* to environmental and genetic changes. The study of regulation requires a precise definition of the term. This definition should distinguish regulation from control, which is a different but related concept, the two concepts often being confounded in the literature. Further, it is necessary to develop methodologies to describe regulatory processes in a quantitative and unambiguous manner. These two aspects are addressed in this thesis. Regulation Analysis, a method to describe the regulation of enzyme rates in a quantitative manner, is refined, extended and used to propose both a precise definition of regulation and a methodology for its study in metabolic systems.

The study of regulation is an endeavor unique to biology. First, it involves the study of systems with a complexity that is unparalleled in the inanimate realm. Second, these complex systems are adaptive. For instance, metabolism responds to environmental perturbations by changing its system properties (fluxes and metabolite concentrations in this case). Frequently, the adapted state cannot be explained by the kinetic properties of the system before the perturbation because also these kinetic properties are actively modulated by the cell. These modulations are determined by yet unknown drives and constraints.

In the following sections, the main sources of metabolic complexity are outlined together with a description of several methodologies that have been developed to study various aspects of this complexity.

1.1 The Complexity of Metabolic Networks

One aspect hindering the understanding of metabolic systems and their regulation is their complexity. Metabolic systems comprise a large number of enzyme-catalyzed reactions that interact non-linearly through metabolic intermediates. In this section, these two sources of complexity, *i.e.* the topology and the non-linearity of the interactions, are described.

1.1.1 Size and structural organization of metabolic networks

Metabolic networks typically comprise hundreds of metabolites involved in a larger number of reactions (*cf.* Palsson, 2006). The number of reactions in which metabolites participate is not uniformly or normally distributed (Edwards & Palsson, 1999; Fell & Wagner, 2000; Wagner & Fell, 2001). Instead, a small number of metabolites is involved in a large number of reactions, while a larger number of metabolites is involved in only a few reactions. The distribution follows a power law (Fell & Wagner, 2000; Wagner & Fell, 2001). Networks with this type of connectivity distribution are called "small-world" networks and share the property that any two nodes are separated by a small number of links.

The biological significance of the small-world architecture of metabolic networks is unclear. Barabasi & Albert (1999) showed that one way of constructing scale free networks is by accretion, where new nodes (*e.g.* metabolites) are connected (*e.g* via reactions) preferentially to nodes that are already highly connected. Such a construction algorithm could have taken place in the early evolution of life (Fell & Wagner, 2000; Wagner & Fell, 2001). On the other hand, Watts & Strogatz (1998) showed that the small-world architecture speeds up the transition between steady-states after a perturbation. Fell and Wagner, however, warn that metabolic dynamics are more complicated than suggested by the simple kinetics used by Watts and Strogatz (Fell & Wagner, 2000; Wagner & Fell, 2001).

1.1.2 Non-linearity of enzyme kinetics

The great majority of chemical reactions in metabolic networks is catalyzed by enzymes. In contrast to the linear kinetics observed in chemical kinetics, the rates of enzyme-catalyzed reactions depend non-linearly on the concentrations of substrates and products and exhibit saturation at high concentrations (Fell, 1997).

The simplest kinetic equation that has been shown to adequately describe the *in vitro* kinetic behavior of some enzymes is the reversible Michaelis-Menten equation:

$$v = \frac{\frac{V_{max}}{K_{m,S}}\left(S - \frac{P}{K_{Eq}}\right)}{1 + \frac{S}{K_{m,S}} + \frac{P}{K_{m,P}}} \tag{1.1}$$

where V_{max} is the rate of catalysis at very high (infinite) concentrations of the substrate S, and in the absence of the product P; K_{Eq} is the equilibrium constant of the chemical reaction, K_m is the Michaelis-Menten constant (and equals the substrate concentration at which the enzyme is half-saturated with substrate in the absence of product), and the subscripts S and P refer to the substrate and product respectively.

The dependence of the enzyme rate on the concentration of the substrate and the product as described by Eq. (1.1) is shown in Figure 1.1. The relation between S and the rate is hyperbolic with an asymptote located at V_{max}. The curvature of the hyperbola depends upon $K_{m,S}$ and upon the concentration of the product. This curvature can be further modified by inhibitors. Description of the latter modification

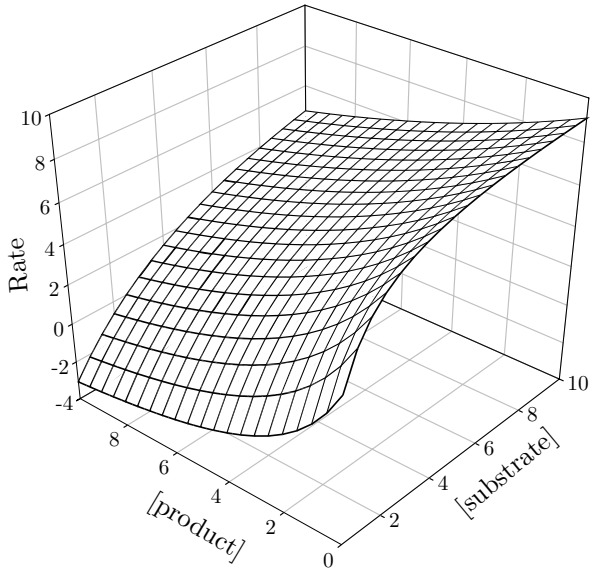

Figure 1.1: An instance of a reversible Michaelis-Menten rate equation. The rate dependence upon the concentration of substrate and product given by Eq. (1.1) is shown ($V_{max} = 10$, $K_{Eq} = 4$, $K_{m,S} = 1$, $K_{m,P} = 2$). Modified from Fell (1997).

requires inclusion of the inhibitor concentration and one or two inhibition constants (depending on the type of inhibition) describing its binding affinity to the enzyme.

Most enzymes have, however, more than one substrate and product. Approximately three quarters of the enzymes reported in public databases have two substrates (Fell, 1997). The kinetics of most of these enzymes can be adequately described by variants of Eq. (1.1) that include concentrations and affinity constants for two substrates and products and, often, the concentrations and affinity constants of one or more inhibitors. These variants are far more complicated than Eq. (1.1) comprising 10 or more kinetic constants (*e.g.* Segel, 1993; Cornish-Bowden, 1995) but are still hyperbolic functions of the substrate concentrations.

A significant minority of enzymes exist that do not show hyperbolic dependencies of the rate as function of substrate concentrations (Fell, 1997). The most common non-hyperbolic enzymes are described by cooperative kinetics comprising several, interacting binding sites. The key characteristic of these descriptions is that the binding affinity of one site depends on whether the other sites are occupied. In the case of positive cooperativity, the affinity for the substrate is enhanced when other binding sites are occupied. Positive cooperativity is diagnosed by a sigmoidal dependence of the rate upon the concentration of the substrate (*cf.* Figure 1.2).

In the 1950s, allosteric inhibition was discovered. A group of enzymes at the beginning of metabolic pathways were found to be inhibited by metabolites further downstream. The significance of this was that the inhibitor bore no chemical re-

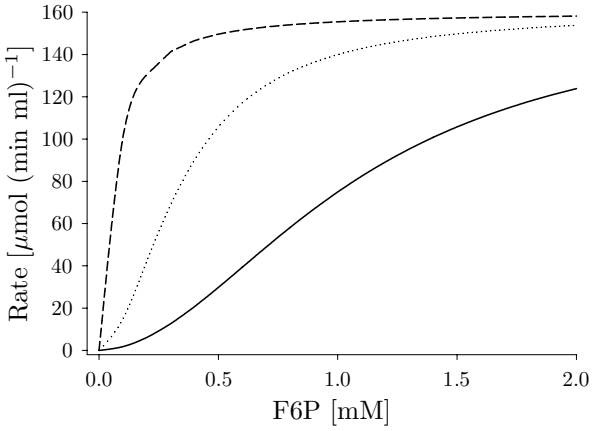

Figure 1.2: An instance of a cooperative kinetics with an allosteric activator. The rate dependence of phosphofructokinase (PFK) on its substrate fructose-6-phosphate (F6P) is shown for different concentrations of PFK's activator frucose-2,6-bisphosphate (F26P). Dashed line ($F26P = 1\mu M$), dotted line ($F26P = 5\mu M$) and solid line ($F26P = 100\mu M$). Modified from Teusink *et al.* (2000).

semblance to either substrates or products and therefore was expected to act on a site other than the active site (Fell, 1997). Monod, Changeux and Jacob proposed a number of generalizations based on the available evidence on allosteric enzymes (Monod *et al.*, 1963): i) allosteric enzymes are multimeric, ii) the modifier (inhibitor or activator) acts by binding to the enzyme at a site different from the active site (these authors coined the term allosteric), iii) in general, the rate dependence on at least one of the substrates is sigmoidal, and iv) in most cases, the modifier affects the half-saturation constant of the enzyme (*cf.* Figure 1.2).

The non-linearity and complexity of enzyme kinetics precludes analytical solutions of metabolic balances even in simple models including only a few reactions. When enzyme kinetics is considered jointly with the magnitude and connectivity of real metabolic networks, the complexity of the description is greatly increased. Only numerical solutions are feasible and multiple solutions for the same parameter set may exist. At the same time, the description is mined with uncertainties concerning not only the kinetic mechanisms and parameters describing enzyme-catalyzed reactions but even the precise topology of the network. In order to identify and evaluate the gravity of these uncertainties, the system's description needs to be explored extensively. The difficulties involved in this exploration increase with the complexity of the system's description. The complexity of the description, in turn, increases with the accuracy with which the description aims to mimic the real system. This is perhaps one of the greatest challenges of contemporary biology, the development of methods and approaches to study the complexity of living systems and ways to explore these descriptions so that they can be confronted with the biological reality.

1.2 Theoretical Approaches to the Study of Metabolism

In the previous section, two important sources of the complexity of metabolic systems were described. In this section, some of the theoretical methodologies that are used to study those aspects of metabolic networks are outlined. The theoretical approaches to the study of metabolism are based on adaptations of methodologies originally developed in non-biological disciplines such as physics, engineering and economics. An important step in those methodologies is the construction of mathematical models. These are simplifying abstractions of reality that serve as complex hypotheses enounced in an unambiguous and quantitative manner.

1.2.1 The mathematical description of metabolic systems

The mathematical description of metabolic systems is often performed by means of time-dependent balance equations of the metabolite pools in terms of the fluxes that produce or remove them (Heinrich *et al.*, 1977).

$$\frac{\mathrm{d}x_i}{\mathrm{d}t} = \sum_{j=1}^{m} c_{ij}\, v_j, \qquad i = 1, \ldots, n \tag{1.2}$$

where x_i represents the concentration of a metabolite i, n the number of different metabolite species, v_j the rates of enzymes producing or removing the metabolite and m the number of reactions in the system. c_{ij} represents the stoichiometric coefficient for a metabolite i in reaction j. The stoichiometric coefficient will be positive if the reaction j produces the metabolite, negative if it removes it and zero if the metabolite does not participate in reaction j. The system of n equations in Eq. (1.2) can alternatively be expressed in matrix notation as:

$$\frac{\mathrm{d}\mathbf{x}}{\mathrm{d}t} = \mathbf{N} \cdot \mathbf{v} \tag{1.3}$$

here $\mathbf{x}$ represents a vector with the concentrations of all metabolites in the system, $\mathbf{N}$ is the stoichiometric matrix containing all the stoichiometric coefficients (encoding the structure or topology of the metabolic network), and $\mathbf{v}$ is a vector containing the values of the rates of all reactions in the system. Since $\mathbf{v}$ depends on $\mathbf{x}$, Eq. (1.3) represents a system of ordinary differential equations.

The structure of metabolic networks

Knowledge of the structure of metabolic networks is prerequisite to any simulation of biochemical networks (Heinrich *et al.*, 1977). The structure of a metabolic network is determined by the stoichiometry of reactions and by the presence and specificity of its enzymes. This information is encoded in the stoichiometric matrix, $\mathbf{N}$. The rows of $\mathbf{N}$ represent metabolite balances and its columns the metabolites that participate in each reaction. If the concentrations of metabolites do not change in time, the system

is said to be in steady state. In this condition the system of differential equations Eq. (1.3) is reduced to a system of algebraic equations:

$$\mathbf{N} \cdot \mathbf{v} = \mathbf{0} \tag{1.4}$$

Although in general the rate equations that determine the rates in the vector $\mathbf{v}$, are non-linear with respect to metabolite concentrations and kinetic parameters, the system of equations Eq. (1.4) is linear if the rates (and not the metabolite concentrations) are considered. Then, Eq. (1.4) can be studied using common functions of linear algebra (*e.g.* Strang, 1986).

Non-zero $\mathbf{v}$ vectors satisfying Eq. (1.4) exist only if $\mathbf{N}$ contains linearly dependent columns, *i.e.* if the rank of $\mathbf{N}$ is smaller than the number of reactions in the system. The linear dependencies among the columns of $\mathbf{N}$ can be identified by computing its null space. The dimension of the null space is the number of columns minus the rank of $\mathbf{N}$ ($m - rank$) and it represents the number of linearly independent vectors $\mathbf{v}$ that are solutions to Eq. (1.4). Any combination of rates in $\mathbf{v}$ that satisfies Eq. (1.4) can be computed as a linear combination of the columns of the null space. Therefore, the null space spans all possible rate combinations that result in a steady state (Strang, 1986).

Also of interest are the linear dependencies among the rows of $\mathbf{N}$. These can be identified by computing the null space of the transpose of $\mathbf{N}$, also called the left null space of $\mathbf{N}$ (Strang, 1986). The existence of linear dependent metabolite balances (encoded in the rows of $\mathbf{N}$) means that linear combinations of these metabolites are constant and that they share a common chemical moiety that neither enters nor leaves the network (Heinrich & Schuster, 1996). As in the case of the null space, the dimensions of the null space of the transpose of $\mathbf{N}$ equals the number of rows minus the rank of $\mathbf{N}$ ($n - rank$) and its columns represent independent conserved moieties.

Applications of structural analysis. The structure of metabolic networks, as encoded in the stoichiometric matrix, can be combined with the measurement of external fluxes to calculate the magnitudes of internal fluxes. In the 1980s, stoichiometric models of the central carbon and free-energy metabolism of *Saccharomyces cerevisiae* (Bonnet *et al.*, 1980) and *Escherichia coli* (Holms, 1986) were constructed. These models were used to compute the intracellular fluxes from measured in- and effluxes.

The stoichiometry of metabolic networks imposes definite limits to the material efficiency of metabolic processes. The amount of a byproduct or biomass produced per unit of substrate consumed has a maximum value that is imposed by the stoichiometry of the network. Considerable effort has been invested in the calculation of maximal biomass yields (*cf.* Stouthamer, 1979). A prerequisite for maximal biomass yield calculations is the determination of the biomass composition in terms of polymers (Lange & Heijnen, 2001, and references therein). For simplicity, biomass is commonly described as consisting of five groups of macromolecules: proteins, carbohydrates, lipids, RNA and DNA. Together with water and metals these macromolecules are the constituents of biomass (Lange & Heijnen, 2001). The biomass composition can be combined with the average polymer compositions in terms of monomers (*i.e.* amino acids, sugars, nucleotides, *etc.*) and with the biosynthetic pathways of these monomers to calculate an assimilation equation describing the synthesis of biomass from mineral substrates.

An assimilation equation for *S. cerevisiae* growing anaerobically on glucose was computed by Verduyn *et al.* The stoichiometric coefficients of this assimilation equation were adjusted to accommodate the observed biomass yield (Verduyn *et al.*, 1990b).

Based on the assimilation equation and previously published estimates of ATP requirements for the polymerization of macromolecules, Verduyn *et al.* summarized the known processes involved in ATP utilization and calculated a 'theoretical' ATP yield of 28.3 g biomass (mol ATP)$^{-1}$ (Verduyn *et al.*, 1990a). An experimental estimation of the ATP yield was made based upon an ATP balance. The ATP production was estimated to be equal to the specific production of ethanol minus the specific production of glycerol. ATP production or consumption due to the formation of other byproducts was neglected on the basis of their low fluxes. The experimental estimate reported was 15.8 g biomass (mol ATP)$^{-1}$, which is much lower than the theoretical estimate quoted above (Verduyn *et al.*, 1991). This large discrepancy between the theoretical and experimental estimations of ATP yields has also been reported for other microorganisms (Stouthamer, 1979). The gap is assumed to be filled by additional ATP utilizing processes such as the transport of metabolites or macromolecules across intracellular membranes, the proofreading of polymerization reactions, the degradation of proteins and mRNA and by signal transduction pathways. However, the quantification of the amount of ATP consumed by these processes is very difficult and little progress has been made in their estimation (Verduyn *et al.*, 1990a).

Constraint-based models and linear programming. Linear metabolite balances can be further constrained by including inequalities in the linear equations indicating the presence of irreversible reactions. These inequalities form a plane in the flux space that intersects the null space dividing the latter into two cones, one of which is compatible with the positive direction of irreversible reactions (Clark, 1980; Schuster *et al.*, 2000; Schilling *et al.*, 2000).

Savinell & Palsson (1992a,b) proposed the assumption that the biomass yield is maximized. They used Linear Programming to choose among alternative flux distributions in underdetermined metabolic networks. This approach has been employed to predict growth and by-product formation rates (*e.g.* Varma & Palsson, 1994; van Gulik & Heijnen, 1995) and for the estimation of the P/O ratio and growth-related maintenance (Vanrolleghem *et al.*, 1996). Constrained stoichiometric models that include the stoichiometric relations of all known metabolic reactions of a particular organism have been constructed and optimized for biomass yield (Edwards & Palsson, 1999, 2000; Schilling *et al.*, 2002; Famili *et al.*, 2003). Despite the "genome-scale" of these network reconstructions, the gap between theoretical and experimental ATP yields remained. However, once these genome-scale models are fitted by introducing a fixed ATP consuming flux to accommodate the experimental ATP yield gap (as proposed by Verduyn *et al.* (1991)), and maximized for biomass yield, they reproduce well the changes in specific fluxes observed in chemostat cultures with varying dilution rates. However, when flux changes as functions of dilution rates deviate from linearity, as in the case of aerobic glucose limited cultures of *S. cerevisiae,* the models fail to reproduce the experimental results (Famili *et al.*, 2003).

The dynamics of metabolic systems

The dynamics of metabolic systems are often described by systems of differential equations that describe metabolite balances in time, Eqs. (1.2) and (1.3). Most often it is assumed that metabolites are homogenously distributed in space (which gives rise to ordinary differential equations) and that enzymes are internally at steady state (*i.e.* the kinetics of the formation of enzyme-metabolite complexes are not included explicitly) (Heinrich *et al.*, 1977).

Stability analysis. If the environmental conditions are stable, metabolic systems often evolve towards a steady state, which is defined by constant values of flux and metabolite concentrations (Heinrich *et al.*, 1977). Steady states may be dynamically stable or unstable. A steady state is globally stable if the system returns to its original steady state after any perturbation. Global stability is, however, very hard to identify (Heinrich *et al.*, 1977). Only the evaluation of local stability will be discussed here. A steady state is said to be locally stable if the system returns to its original steady state after a small perturbation.

The procedure to evaluate the local stability of a steady-state will be illustrated for a two-component system. The local stability of systems of higher dimensions is analyzed in essentially the same way (*cf.* Hubbard & West, 1995).

Consider a system of two non-linear differential equations:

$$\begin{cases} \dfrac{\mathrm{d}x}{\mathrm{d}t} = f(x,y) \\ \dfrac{\mathrm{d}y}{\mathrm{d}t} = g(x,y) \end{cases} \tag{1.5}$$

for which it's possible to find variable values x^0 and y^0 that satisfy the equilibrium condition, *i.e.*:

$$\begin{cases} f(x^0,y^0) = 0 \\ g(x^0,y^0) = 0 \end{cases} \tag{1.6}$$

A linear approximation to the system of non-linear differential equations Eq. (1.5) is made by a first order Taylor expansion arround the equilibrium-state:

$$\begin{cases} \dfrac{\mathrm{d}x}{\mathrm{d}t} \approx f(x^0,y^0) + \left.\dfrac{\partial f}{\partial x}\right|_{\substack{x=x^0\\y=y^0}} \cdot (x-x^0) + \left.\dfrac{\partial f}{\partial y}\right|_{\substack{x=x^0\\y=y^0}} \cdot (y-y^0) \\ \dfrac{\mathrm{d}y}{\mathrm{d}t} \approx g(x^0,y^0) + \left.\dfrac{\partial g}{\partial x}\right|_{\substack{x=x^0\\y=y^0}} \cdot (x-x^0) + \left.\dfrac{\partial g}{\partial y}\right|_{\substack{x=x^0\\y=y^0}} \cdot (y-y^0) \end{cases} \tag{1.7}$$

The system of equations Eq. (1.7) can be expressed in matrix form as:

$$\begin{bmatrix} \dot{x} \\ \dot{y} \end{bmatrix} \approx \left. \begin{bmatrix} \dfrac{\partial f}{\partial x} & \dfrac{\partial f}{\partial y} \\ \dfrac{\partial g}{\partial x} & \dfrac{\partial g}{\partial y} \end{bmatrix} \right|_{\substack{x=x^0\\y=y^0}} \cdot \begin{bmatrix} x-x^0 \\ y-y^0 \end{bmatrix} \tag{1.8}$$

where $\begin{bmatrix} \frac{\partial f}{\partial x} & \frac{\partial f}{\partial y} \\ \frac{\partial g}{\partial x} & \frac{\partial g}{\partial y} \end{bmatrix}$ is the Jacobi matrix.

With the change of variables:

$$\begin{aligned} u = (x - x^0) \qquad \therefore \qquad \frac{\mathrm{d}u}{\mathrm{d}t} = \frac{\mathrm{d}x}{\mathrm{d}t} \\ v = (y - y^0) \qquad \therefore \qquad \frac{\mathrm{d}v}{\mathrm{d}t} = \frac{\mathrm{d}y}{\mathrm{d}t} \end{aligned} \tag{1.9}$$

the system of *linear* differential equations Eq. (1.7) may be expressed as:

$$\begin{bmatrix} \dot{u} \\ \dot{v} \end{bmatrix} \approx \begin{bmatrix} a & b \\ c & d \end{bmatrix} \cdot \begin{bmatrix} u \\ v \end{bmatrix} \tag{1.10}$$

where a, b, c and d represent the partial derivatives $\partial f/\partial x$, $\partial f/\partial y$, $\partial g/\partial x$ and $\partial g/\partial y$, respectively, evaluated in the equilibrium-state.

Systems of linear differential equations, such as Eq. (1.10) have the well known solution:

$$\begin{bmatrix} u \\ v \end{bmatrix} = \begin{bmatrix} C_u \\ C_v \end{bmatrix} \cdot e^{\lambda\, t} \qquad \therefore \qquad \begin{bmatrix} \dot{u} \\ \dot{v} \end{bmatrix} = \lambda \cdot e^{\lambda\, t} \cdot \begin{bmatrix} C_u \\ C_v \end{bmatrix} \tag{1.11}$$

Substituting Eq. (1.11) into Eq. (1.10) and using matrix notation one obtains:

$$\lambda \cdot \mathbf{c} = \mathbf{A} \cdot \mathbf{c} \qquad \therefore \qquad (\mathbf{A} - \lambda \cdot \mathbf{I}) \cdot \mathbf{c} = 0 \tag{1.12}$$

where λ represents the eigenvalues of the Jacobi matrix and $\mathbf{c}$ its eigenvectors, $\mathbf{A}$ is the matrix of evaluated partial derivatives shown in Eq. (1.10), *i.e.* the Jacobi matrix evaluated in the equilibrium-state, and $\mathbf{I}$ is the identity matrix.

Non-trivial solutions for Eq. (1.12) exist (*i.e.* solutions with $\mathbf{c} \neq 0$) if the matrix $(\mathbf{A} - \lambda \cdot \mathbf{I})$ has linearly dependent columns, that is, if the matrix's determinant is zero. This fact is used to compute the eigenvalues by solving:

$$\det |\mathbf{A} - \lambda \cdot \mathbf{I}| = 0 \tag{1.13}$$

which yields the characterisitic equation, which for 2 component systems is:

$$a_2\, \lambda^2 + a_1\, \lambda + a_0 = 0 \tag{1.14}$$

Equation (1.14) has two roots, which are the eigenvalues of the Jacobi matrix. If both eigenvalues are real and positive, the equilibrium is locally unstable (called a source), if they are both real and negative, the equilibrium is locally stable (called a sink). It may happen that the eigenvalues are real and have opposite signs. Then the equilibrium is a saddle point (a sink in one dimension and and source in the other). In the case where one or more eigenvalues are zero, the system is said to be critical, statements on the equilibrium's stability can only be made based on consideration of quadratic of higher terms in the Taylor expansion.

The eigenvalues of the Jacobi matrix may be complex, in which case the system will oscillate. The stability of the equilibrium, however, depends only upon the real part of the complex eigenvalues. The equilibrium is a source if the real part of the eigenvalues is possitive and a sink if its negative. If the eigenvalues are complex and their real part is zero, the system is said to be upon a Hopf bifurcation. In such cases, the stability of the system can be changed through small adjustments of one of the parameters in Eq. (1.5). Often, when the equilibrium transits form a sink to a source through parameter adjutments; in the vecinity of the Hopf bifurcation, a special kind of sink is formed that is called a "limit cycle" in which the system shows stable oscillations.

The formation of a limit cycle does not depend only upon the *local* stability of the equilibrium but also on the gobal stability of the non-linear system of differential equations Eq. (1.5). Limit cycles are formed near Hopf biffurcations where, locally, the equilibrium is a weak source but globally its a strong sink. Trajectories from without the limit cycle have the tendency to converge to its center but, locally, the center is a source. Therefore the trajectories are "trapped" in the limit cycle.

Limit cycles have been observed experimentally as autonomous biochemical oscillations, these were first observed in intact yeast glycolysis with typical periods in the order of minutes (Ghosh & Chance, 1964; Chance *et al.*, 1964a,b). Thereafter, glycolytic oscillations have been studied in cell-free extracts (Hess & Boiteux, 1968).

Metabolic Control Analysis. If a steady state is locally stable, it is possible to study the sensitivity of its system properties towards changes in the system parameters using Metabolic Control Analysis (MCA). MCA is a sensitivity analysis for metabolic systems. It relates sensitivities of system components (*i.e.* enzymes) to the sensitivities of metabolic system properties (*i.e.* fluxes and metabolite concentrations) (Kacser & Burns, 1973; Heinrich & Rapoport, 1974). The former sensitivities are called elasticities. They quantify the extent to which a property of a component (*e.g.* the rate of catalysis of a given enzyme) is changed in response to a change in a parameter or variable that affects that component directly. The latter sensitivities are called control coefficients and they quantify the extent to which the system properties of the metabolic system change in response to a change of a parameter of the system. The definitions of elasticity and control coefficients and the relations between them are more easily introduced with the aid of a simple example. The following derivation is based on Reder's structural approach to MCA (Reder, 1988). In this example, matrix notation has been avoided for the sake of mathematical ease.

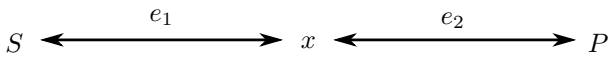

Figure 1.3: Linear pathway with one intermediate.

Consider a linear pathway composed of two enzymes converting S into P with a unique intermediate x (Figure 1.3). In the steady state, the following equations are satisfied:

$$\frac{\mathrm{d}x}{\mathrm{d}t} = 0 = v_1 - v_2 \quad \therefore \quad v_1 = v_2 \equiv J \tag{1.15}$$

The system can be perturbed by an infinitesimal change in e_1. This change affects v_1 both directly as well as through the change in the intermediate x, while it affects v_2 through x. Thus:

$$\frac{\mathrm{d}\left(\frac{\mathrm{d}x}{\mathrm{d}t}\right)}{\mathrm{d}e_1} = 0 = \frac{\partial v_1}{\partial e_1} + \frac{\partial v_1}{\partial x}\frac{\mathrm{d}x}{\mathrm{d}e_1} - \frac{\partial v_2}{\partial x}\frac{\mathrm{d}x}{\mathrm{d}e_1} \tag{1.16}$$

Normalizing Eq. (1.16) so that it becomes dimensionless, results in:

$$0 = \frac{\partial \ln v_1}{\partial \ln e_1} + \frac{\partial \ln v_1}{\partial \ln x}\frac{\mathrm{d}\ln x}{\mathrm{d}\ln e_1} - \frac{\partial \ln v_2}{\partial \ln x}\frac{\mathrm{d}\ln x}{\mathrm{d}\ln e_1} \tag{1.17}$$

In the MCA notation Eq. (1.17) is re-written as:

$$0 = \varepsilon_{e_1}^{v_1} + \varepsilon_x^{v_1} C_{e_1}^x - \varepsilon_x^{v_2} C_{e_1}^x \tag{1.18}$$

Equation (1.18) shows that the elasticities ε, are scaled *partial* derivatives of component properties (here enzyme rates) with respect to system variables or parameters (here the enzyme concentration in $\varepsilon_{e_1}^{v_1}$ or the concentration of x in $\varepsilon_{v_1}^x$). In contrast, control coefficients C are scaled *total* derivatives of metabolic system properties [in Eq. (1.18) the concentration of x] with respect to system parameters (here enzyme concentrations).

Because most enzyme rates are proportional to the concentration of enzyme, the first term on the right hand side of Eq. (1.17) usually equals unity. Therefore Eq. (1.18) can alternatively be written as:

$$C_{e_1}^x = -\frac{1}{\varepsilon_x^{v_1} - \varepsilon_x^{v_2}} \tag{1.19}$$

Equation (1.19) illustrates the fact that sensitivities of system properties (*i.e.* control coefficients) can be written as functions of the sensitivities of system components (*i.e.* elasticities). *i.e.* the properties of the components together determine the properties of the system.

With the definition of flux J in Eq. (1.15) the total derivative of the flux with respect to e_1 can be computed:

$$\frac{\mathrm{d}J}{\mathrm{d}e_1} = \frac{\mathrm{d}v_1}{\mathrm{d}e_1} = \frac{\partial v_1}{\partial e_1} + \frac{\partial v_1}{\partial x}\frac{\mathrm{d}x}{\mathrm{d}e_1} \tag{1.20}$$

which, after scaling, can be expressed in MCA notation as:

$$C_{e_1}^J = 1 + \varepsilon_x^{v_1} C_{e_1}^x \tag{1.21}$$

The equation above shows that the flux control coefficient,$C_{e_1}^J$, can be written as a function of the elasticity of the enzyme's rate towards the concentration of x and the concentration control coefficient of x with respect to the concentration of the first enzyme. Combining Eqs. (1.19) and (1.21) one obtains:

$$C_{e_1}^J = 1 - \frac{\varepsilon_x^{v_1}}{\varepsilon_x^{v_1} - \varepsilon_x^{v_2}} \tag{1.22}$$

As in the case of Eq. (1.19), Eq. (1.22) illustrates the fact that a control coefficient can be expressed as a function of the elasticity coefficients. Eq. (1.22) also shows that a change of an enzyme concentration initially produces a proportional change in the enzyme rate that is counteracted by the response of the system to this perturbation [as described the second term on the right hand side of Eq. (1.22)].

The procedure described above can be repeated by perturbing e_2 which results in the following control coefficients:

$$C^x_{e_2} = \frac{1}{\varepsilon^{v_1}_x - \varepsilon^{v_2}_x} \tag{1.23}$$

$$C^J_{e_1} = 1 + \varepsilon^{v_2}_x C^x_{e_2} = 1 + \frac{\varepsilon^{v_2}_x}{\varepsilon^{v_1}_x - \varepsilon^{v_2}_x} \tag{1.24}$$

The development of MCA was not limited to the adaptation of sensitivity analysis to metabolic systems but also discovered theorems of metabolic control (Kacser & Burns, 1973; Heinrich & Rapoport, 1974). The proofs of these theorems for general systems are beyond the scope of this introduction. The reader is referred to Reder (1988) for a general proof of all MCA theorems. In this section, MCA theorems will be enounced and then shown to apply to the system illustrated here.

The first set of theorems contains the summation theorems:

$$\sum_{i=1}^{n} C^J_{e_i} = 1 \tag{1.25}$$

$$\sum_{i=1}^{n} C^J_{e_i} = 0 \qquad\qquad j = 1, \ldots, m \tag{1.26}$$

Equation (1.25) enounces the summation theorem for flux control coefficients, which can easily be shown to hold for our example by summing Eqs. (1.22) and (1.24). This theorem implies that if all flux control coefficients are non-negative, then they must all be smaller than 1. Therefore, in linear pathways control coefficients are expected to be all smaller than 1. Flux control coefficients bigger than unity can only occur if negative control coefficients also occur, which may happen, for instance, in branched pathways.

Equation (1.26) enounces the summation theorem for concentration control coefficients that for our example can be shown to hold by summing Eqs. (1.19) and (1.23). This theorem imposes a balance between positive and negative concentration control coefficients and illustrates the fact that if consuming and producing reactions are changed proportionally, the concentration of the intermediate must remain unchanged.

The second set of MCA theorems are the connectivity theorems.

$$\sum_{i=1}^{n} C^J_{e_i} \varepsilon^{v_i}_{x_j} = 0 \qquad\qquad j = 1, \ldots, m \tag{1.27}$$

$$\sum_{i=1}^{n} C^{x_j}_{e_i} \varepsilon^{v_i}_{x_k} = -\delta_{jk} \qquad\qquad \delta_{jk} \equiv \begin{cases} 1, \text{ if } j = k \\ 0, \text{ if } j \neq k \end{cases} \tag{1.28}$$

Equation (1.27) enounces the connectivity theorem for flux control coefficients. It implies that if two enzymes in a linear pathway are connected via a common metabolite, the relative control that those enzymes have on the pathway's flux is determined by their relative elasticities towards that metabolite. Eq. (1.27) can alternatively be interpreted as showing the fact that the sum of flux responses ($R^J_{x_j} = C^J_{e_i}\,\varepsilon^{v_i}_{x_j}$) of all individual enzymes to a change in the concentration of their common metabolite x_j is zero. This means that the flux through the pathway cannot be changed by perturbations of internal metabolites, which are variables, not parameters of the system.

The second connectivity theorem, Eq. (1.28), can be proven true for our example by substituting Eqs. (1.21) and (1.22) into the summation theorem for flux control coefficients, Eq. (1.25). Eq. (1.28) shows that the sum of concentration responses ($R^{x_k}_{x_j} = C^{x_j}_{e_i}\,\varepsilon^{v_i}_{x_k}$) to a change in the concentration of the intermediate x_k are such as to exactly counter act the perturbation.

1.3 Regulation of Metabolic Networks

Section 1.2 described how metabolic system properties (fluxes, metabolite concentrations and control coefficients) are determined by the properties of the components of the system. Another important determinant of these system properties is the interaction of the metabolic network with its environment. It is often observed that living cells adapt to environmental changes by regulating their metabolic system properties. When environmental changes are imposed by Nature or by an experimenter, the interaction between the metabolic network and the environment is perturbed. The perturbation propagates into the network via direct effects on enzymes that are transmitted via intermediate metabolite concentrations, and via indirect effects through signal transduction and gene expression, eventually affecting metabolic fluxes and metabolite concentrations. The direct effect of an external perturbation upon the system properties of a network is constrained by the kinetic properties of the enzymes, *i.e.* the kinetic properties of the enzymes regulate the initial response of the system to an external perturbation. This initial perturbed state is often short-lived. The cell further regulates its fluxes and metabolite concentrations through the modulation of enzyme activities and their kinetic properties.

In this section, efforts to define regulation in a quantitative manner and some hypotheses upon the constraints of regulatory processes are delineated. In a later section (Section 1.4), the mechanisms through which the activities and kinetic properties of enzymes are modulated will be described.

1.3.1 Definitions of regulation

The term "regulation" is not well defined in biology. Arguably, the failure to define regulation unambiguously is one of the difficulties hindering progress in our understanding of biological regulation. In the past, the terms regulation and control were used interchangeably and statements upon regulation and control were often qualitative and vague (Fell, 1997; Sauro, 1989). The term *control* has been given a precise and quantitative definition through the development of Metabolic Control Analysis

(*cf* Fell, 1997). Several efforts to define *regulation* quantitatively are based on or inspired by Metabolic Control Analysis as well.

Westerhoff & Chen (1984) and Westerhoff & Van Dam (1987) focused on the regulation of metabolic fluxes or metabolite concentrations in response to a perturbation of the concentration of a given metabolite, also called a fluctuation. They described the response of the system to the addition of an amount of metabolite x_k as:

$$\frac{\delta y}{y} = \sum_{i=1}^{n} C_{e_i}^{y} \cdot \varepsilon_{x_k}^{v_i} \cdot \frac{\delta x_k}{x_k} \tag{1.29}$$

where y represents a system variable, *i.e.* a metabolic flux or a metabolite concentration. Eq. (1.29) enounces that the response of a system variable y to a fluctuation in the concentration of metabolite x_k depends upon the sensitivities of all enzymes towards the changing metabolite and the sensitivity of the system property y to changes in the rates of those enzymes. These authors noted that if the system was in an asymptotically stable steady-state, the magnitude $\sum_{i=1}^{n} C_{e_i}^{y} \cdot \varepsilon_{x_k}^{v_i}$ should be zero if $y \neq x_k$ and -1 if $y = x_k$. This then constituted a new proof of the connectivity theorems, Eqs. (1.27) and (1.28). The terms $C_{e_i}^{y} \cdot \varepsilon_{x_k}^{v_i}$ in Eq. (1.29) function as internal responses (Westerhoff & Van Dam, 1987) or regulatory strengths (Kahn & Westerhoff, 1993) and they correspond to the Response Coefficients $R_{x_k}^{J\,(\mathrm{or}\,x_j)}$ that were introduced in the Metabolic Control Analysis paragraph in Section 1.2.1

By contrast, Hofmeyr and Cornish-Bowden focused on the regulation by external factors (Hofmeyr & Cornish-Bowden, 1991). They started by defining internal and external parameters. The former are invariant at the time-scale of interest (*e.g.* V_{max}, K_m and K_{eq}) and the latter are environmental (*e.g.* the concentration of the substrate). According to these authors, a system can only be regulated by changes in external parameters, which they call regulators. How effectively a system is regulated depends upon the degree to which the activity of the regulatory enzyme, the enzyme with which the regulator interacts directly, can be altered by the regulator (named its regulability) and on the ability of the regulatory enzyme to transmit the changes to the rest of the system (named its regulatory capacity). In MCA notation, this can be expressed as a response coefficient:

$$R_S^{J(or\,x)} = \varepsilon_S^{v_i}\, C_{v_i}^{J(or\,x)} \tag{1.30}$$

where $R_S^{J(or\,x)}$ is the response coefficient of the flux J or the concentration of the metabolic intermediate x, $\varepsilon_S^{v_i}$ is the elasticity of the regulatory enzyme i, towards the regulator S and quantifies its regulability. $C_{v_i}^{J(or\,x)}$ is the flux or concentration control coefficient of the regulatory enzyme i, it quantifies the regulatory capacity of the regulatory enzyme i.

Sauro approached the study and definition of regulation from a yet different point of view. He foucused on the regulation of enzyme rates by the many factors affecting them (Sauro, 1989). He noted that local rates and fluxes, although quantitatively identical in the steady state, are conceptually very different because fluxes are determined by the whole system whereas local rates are determined by the kinetic properties of a single enzyme and the concentrations of metabolites affecting the rate of that enzyme.

The local rates of enzyme-catalyzed reactions depend upon their capacities, the concentrations of metabolic effectors affecting their rate and the affinity constants that parametrize the strengths with which metabolic effectors interact with the enzymes:

$$v = \varphi(e, \mathbf{x}, \mathbf{k}) \tag{1.31}$$

in which v is the rate of the enzyme of interest, e is the concentration of this enzyme, $\mathbf{x}$ is a vector of concentrations of substrates, products and other metabolic effectors, and $\mathbf{k}$ is a vector of interactions constants of this enzyme with its substrates, products and other metabolic effectors. Sauro defined regulation as the response of the system to changes in its environment and proposes a quantitative description of the regulation of *local* rates. Since not all enzymes interact directly with the environment the regulation of the majority of enzymes will depend only on the concentrations in the vector $\mathbf{x}$ (*i.e.* when only short times after the perturbation are considered). It is then possible to dissect the contributions of the changes of each of the metabolite concentrations in $\mathbf{x}$ to the regulation of the local flux. Sauro's argument is best expounded using his own example. Consider the linear pathway in Figure 1.4. Let the rate of the fifth step

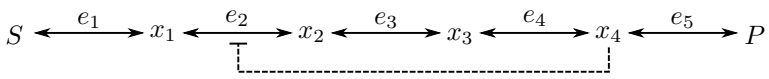

Figure 1.4: Linear pathway with feedback inhibition. Modified from Sauro (1989).

(e_5) be modified by an environmental or genetic perturbation. The total derivative of the local rate with respect to the perturbation of e_5 is given by:

$$\mathrm{d}v_2 = \frac{\partial v_2}{\partial x_1}\frac{\mathrm{d}x_1}{\mathrm{d}e_5}\mathrm{d}e_5 + \frac{\partial v_2}{\partial x_2}\frac{\mathrm{d}x_2}{\mathrm{d}e_5}\mathrm{d}e_5 + \frac{\partial v_2}{\partial x_4}\frac{\mathrm{d}x_4}{\mathrm{d}e_5}\mathrm{d}e_5 \tag{1.32}$$

which after normalization can be written in MCA notation as:

$$\frac{\mathrm{d}v_2}{v_2} = \varepsilon_{x_1}^{v_2} C_{v_5}^{x_1}\frac{\mathrm{d}e_5}{e_5} + \varepsilon_{x_2}^{v_2} C_{v_5}^{x_2}\frac{\mathrm{d}e_5}{e_5} + \varepsilon_{x_4}^{v_2} C_{v_5}^{x_4}\frac{\mathrm{d}e_5}{e_5} \tag{1.33}$$

In the steady state the relative change in the pathway flux ($\mathrm{d}J/J$) equals the change in the local rate ($\mathrm{d}v_2/v_2$). Dividing both sides of Eq. (1.33) by $\mathrm{d}J/J$ gives:

$$1 = \varepsilon_{x_1}^{v_2}\frac{C_{v_5}^{x_1}}{C_{v_5}^{J_2}} + \varepsilon_{x_2}^{v_2}\frac{C_{v_5}^{x_2}}{C_{v_5}^{J_2}} + \varepsilon_{x_4}^{v_2}\frac{C_{v_5}^{x_4}}{C_{v_5}^{J_2}} \equiv {}^{v_5}P_{x_1}^{J_2} + {}^{v_5}P_{x_2}^{J_2} + {}^{v_5}P_{x_4}^{J_2} \tag{1.34}$$

where ${}^{v_5}P_{x_i}^{J_2}$ are the partitioned response coefficients for the metabolite x_i. The partitioned response coefficients sum up to one. Therefore, the contributions of each of the metabolite concentration changes to the regulation of the local rate can be dissected quantitatively.

The definitions of regulation by Westerhoff & Chen (1984), Westerhoff & Van Dam (1987), Kahn & Westerhoff (1993), Hofmeyr & Cornish-Bowden (1991) and Sauro (1989) are mathematically very similar. The concentration response coefficient, Eq. (1.30), equals the partitioned response coefficient times the concentration control

coefficient of the enzyme in question. They are conceptually, however, different. Sauro quantitatively dissects the regulation of local rates by changes in the metabolite concentrations that affect that rate. While the others quantify the regulation of system properties by changes in internal (Westerhoff & Chen, 1984; Westerhoff & Van Dam, 1987; Kahn & Westerhoff, 1993) and external metabolite concentrations (Hofmeyr & Cornish-Bowden, 1991).

Later Hofmeyr proposed another definition of regulation. He defined regulation as the alteration of reaction properties to augment or counteract the mass-action trend in a network of reactions (Hofmeyr, 1995). The response of the system to perturbations of any kind depends both on the intrinsic mass-action trend and the kinetic properties of enzymes. Hofmeyr used Reich and Sel'kov's (Reich & Sel'kov, 1981) argument that any physiologically realizable rate equation can be written as a product of the rate constant, f; a saturation term, θ; and a thermodynamic term, ξ.

$$v = f(e) \cdot \theta(\mathbf{x}, \mathbf{k}) \cdot \xi(\Gamma(\mathbf{x})/K_{Eq}) \tag{1.35}$$

where Γ is the mass-action ratio (*i.e.* the actual ratio of product and substrate concentrations), K_{Eq} is the equilibrium constant of the reaction and e, $\mathbf{x}$ and $\mathbf{k}$ have the same interpretation as in Eq. (1.31). Note that ξ is a function of the displacement of the reaction from equilibrium, it has the form (1-Γ/K_{Eq}) and equals zero at the equilibrium. Translating Eq. (1.35) into logarithmic space and deriving the resulting function with respect to $\ln(x_i)$, where x_i is concentration of a substrate or product (not of an allosteric activator or inhibitor) of the reaction, gives:

$$\varepsilon^{v}_{x_i} = \frac{\mathrm{d}\ln v}{\mathrm{d}\ln x_i} = \frac{\mathrm{d}\ln \theta}{\mathrm{d}\ln x_i} + \frac{\mathrm{d}\ln \xi}{\mathrm{d}\ln x_i} \tag{1.36}$$

Note that since *f(e)* is usually independent of the metabolite concentrations, $\mathrm{d}f/\mathrm{d}x_i$ is zero. For an allosteric effector the last term of Eq. (1.36) is zero. Eq. (1.36) shows that elasticities towards substrates or products can be expressed as the sum of a kinetic term and a thermodynamic term. The latter becomes extremely high whenever the reaction is colise to equilibrium (Westerhoff & Van Dam, 1987). This interesting concept is, however, limited to local properties of metabolic systems (*i.e.* elasticities). For system properties such as flux and concentration control coefficients, it is not possible to dissect the contributions by kinetic and thermodynamic properties.

The last effort to quantify regulation that will be discussed here, is that of Westerhoff (Westerhoff *et al.*, 2000; ter Kuile & Westerhoff, 2001), which is called Regulation Analysis. Like Sauro's method, Regulation Analysis is concerned with the local regulation of enzyme rates. However, it departs form MCA's restriction to infinitesimally small changes through Reich and Sel'kov's identification of a general property of enzyme catalyzed reactions (Reich & Sel'kov, 1981). Because enzymes are catalyst and not substrates, rate equations are usually of the form:

$$v = f(e) \cdot g(\mathbf{x}, \mathbf{k}) \tag{1.37}$$

The important characteristic of Eq. (1.37) is that the two multipliers on the right-hand side are cross-independent. This means that f does not depend upon $\mathbf{x}$ and $\mathbf{k}$,

and g does not depend upon e. $f(e)$ describes the dependency of the rate upon the enzyme concentration, while $g(\mathbf{x},\mathbf{k})$ describes the interaction of the enzyme with the rest of metabolism through metabolite concentrations and the corresponding affinity constants. Eq. (1.37) can be translated into logarithmic space and if two different steady states are considered, the difference between their local steady state rates is then given by:

$$\Delta \ln v = \Delta \ln f(e) + \Delta \ln g(\mathbf{x}, \mathbf{k}) \tag{1.38}$$

The crux of Regulation Analysis is that since the multipliers in Eq. (1.37) are cross-independent, Eq. (1.38) remains valid for small as well as large changes. As already pointed out by Sauro, at steady state the flux J equals the enzyme rate v. Dividing both sides of Eq. (1.38) by the flux change in logarithmic space ($\Delta \ln J$) results in:

$$1 = \frac{\Delta \ln f(e)}{\Delta \ln J} + \frac{\Delta \ln g(\mathbf{x}, \mathbf{k})}{\Delta \ln J} = \rho_h + \rho_m \tag{1.39}$$

in which ρ_h is the 'hierarchical regulation coefficient', quantifying the relative contribution of changes in enzyme concentration to the regulation of its flux, and ρ_m is the 'metabolic regulation coefficient', quantifying the relative contribution of changes in the interaction of the enzyme with the rest of metabolism to the regulation of the the flux. The two regulation coefficients sum up to one (summation theorem for the regulation of flux) implying that determination of one will yield the other automatically. In practice, the hierarchical regulation coefficient is more readily determined, since $f(e)$ usually equals V_{max}, and the V_{max} as well as the flux J through the enzyme can be measured or estimated in most cases. Regulation Analysis introduces the possibility of making unambiguous and quantitative descriptions of the regulation of fluxes through individual enzymes embedded in biochemical networks of any complexity, in response to any number or kind of simultaneous perturbations.

The essential difference between the methods of Sauro and Westerhoff is that the latter includes adaptations through gene expression (ρ_h), while the former does not. Actually, Sauro's analysis can be used to dissect the metabolic regulation coefficient ρ_m into the contributions by each individual metabolite. Since the metabolite concentrations usually do not enter enzymatic rate equations as independent multipliers, Sauro's analysis can only be applied to small changes. Similarly, the hierarchical regulation coefficient ρ_h can be dissected into the contributions by the various processes in the gene-expression cascade. The dissection of the hierarchical regulation coefficient into terms that relate to various levels of gene expression is expounded in Chapter 4.

The different quantitative descriptions of regulation described above are not theories of regulation but ways to describe regulatory processes quantitatively. Regulation is often associated with homeostasis (Fell, 1997; Hofmeyr & Cornish-Bowden, 1991). Hofmeyr and Cornish-Bowden proposed that the performance of a metabolic system can be judged in terms of how sensitively the fluxes respond to external stimuli and to what degree homeostasis in the concentrations of "key" metabolites is maintained. Key metabolites were defined as those that divide the metabolic system in supply and demand blocks (Hofmeyr & Cornish-Bowden, 1991). The reality of such blocks in actual metabolic networks is, however, disputable due the very high connectivity of coenzymes. Fell also proposed that regulatory responses are constrained to maintain homeostasis of all metabolites in the pathway while changing fluxes. He argued

that this is achieved through the simultaneous modulation of all enzyme activities in a pathway. This hypothesis of multi-site modulation has met supporting examples, such as lipogenesis in mice, the urea cycle in rats, and photosynthesis in green plants (Fell & Thomas, 1995). It may be questioned, however, how general this mechanism is, and whether indeed even in these examples all enzyme activities changed in proportion to the flux. This issue is discussed in Chapter 3.

An older, and very different, hypothesis on metabolic regulation is that of economy of protein synthesis, which was taken to imply the modulation of fluxes through modulation of rate limiting steps. Experimental investigations based on MCA suggest that often the control of flux is distributed over many enzymes in a pathway (Groen *et al.*, 1986; Fell, 1992). Therefore, modulation of a single enzyme may be an ineffective mechanism for changing the flux through a pathway. Indeed, attempts to correlate flux changes with changes in single enzyme activities or levels have failed consistently (Daran-Lapujade *et al.*, 2004; Nilsson *et al.*, 2001b; van Hoek *et al.*, 1998b).

1.4 Glycolysis and Carbon Metabolism in *Saccharomyces cerevisiae*

Although the methodological developments reported in this thesis are not limited to any given organism, the experimental investigations focus on the regulatory processes involved in the adaptation of the glycolytic pathway in *Saccharomyces cerevisiae* to environmental and genetic perturbations. Particular emphasis is put on conditions and perturbations that are related to the industrial production and the utilization of bakers' yeast.

The yeast *Saccharomyces cerevisiae* is the best-characterized eukaryote today. The extraordinary attention devoted to this yeast by the scientific community has undoubtedly been motivated by its association with the wine, beer and bakers' industries (*e.g.* Gancedo & Serrano, 1989), while the fruits of scientific inquiry in turn constitute further incentives to new research on this organism.

There are also intrinsic advantages favouring *S. cerevisiae* as a model organism. First, it is a eukaryote and therefore to a large extent representative of other eukaryotes, including the cells of multicellular organisms. Second, it is a non-pathogenic microorganism that grows rapidly and reproducibly on rich as well as mineral media. Third, it is particularly suited for genetic studies for numerous reasons. It grows as dispersed cells, which facilitates mutant isolation. Its haploid and diploid states are stable, which enables the isolation and expression of mutants as well as complementation tests. Plasmids can be introduced as replicating molecules or integrated into the genome. Plasmid integration proceeds exclusively via homologous recombination. Finally, bakers' yeast is viable with numerous markers (Sherman, 1997).

1.4.1 The Glycolytic Pathway in *Saccharomyces cerevisiae*

S.cerevisiae is a heterotrophic organism that derives its free energy from the oxidation of carbohydrates. Consequently, carbon and energy metabolisms are closely interconnected in this organism (Gancedo & Serrano, 1989). Glucose is the preferred growth

substrate for many microorganisms and ideed all yeast species studied so far can grow on glucose (Pronk *et al.*, 1996).

Sugars do not permeate freely through lipid membranes. Therefore, the first catalysed step in glucose metabolism is its transport across the plasmamembrane (Kotyk, 1967; Lang & Cirillo, 1987; Fuhrmann *et al.*, 1989; Lagunas, 1993). In *S. cerevisiae*, glucose transport is facilitated by a family of hexose transporters (HXT1-HXT17), which have different affinities for glucose (Wieczorke *et al.*, 1999; Boles & Hollenberg, 1997; Kruckeberg, 1996). Of the seven dominant hexose transporters, HXT1 and HXT3 have a low affinity (50 – 100 mM), HXT6 and HXT7 have a higher affinity (1-2 mM) and HXT2 and HXT4 have intermediate affinities (Reifenberger *et al.*, 1997). Despite the large number of different transporters, glucose transport can usually be satisfactorily described by a high affinity and a low affinity component (Bisson & Fraenkel, 1983). The appearance of these components and the expression of the HXT genes depends upon the extracellular glucose concentration and is modulated during growth (Diderich *et al.*, 1999a; Walsh *et al.*, 1994). Kinetic models of glycolysis in *S. cerevisiae* suggest that the glucose transporters have a high control upon the glycolytic flux (Galazzo & Bailey, 1990; Reijenga *et al.*, 2005). These suggestions are supported by experimental measurements of the control coefficient of the glucose transporter in a related yeast species, *Saccharomyces bayanus* (Diderich *et al.*, 1999b).

The glycolytic pathway as is operates in bakers' yeast is shown in Figure 1.5. The conversion of glucose into ethanol is catalysed by 12 enzymes and involves 17 metabolites. The rate of fermentation is expected to depend upon the kinetic properties of and the interactions between these enzymes (Boiteux & Hess, 1981) and upon the thermodynamic properties of the chemical reactions catalysed by these enzymes. The glycolytic and fermentative pathways of *S. cerevisiae* are perhaps the most extensively studied metabolic pathways. Research on these pathways gave birth to the discipline of Biochemistry in the second half of the 19^{th} century with the passionate controversy about the mechanism of alcoholic fermentation (Gancedo & Serrano, 1989). All the enzymes involved in alcoholic fermentation have been characterized kinetically *in vitro* and the genes encoding them have been identified. One might then expect that, given the engineering possibilities introduced by molecular biology, it should be possible to rationally engineer the fermentative flux. Such efforts, however, have so far proven fruitless (Schaaff *et al.*, 1989; Heinisch, 1986; van Hoek *et al.*, 1998a; Hauf *et al.*, 2000).

Traditional approaches to flux engineering were devoted to the search of rate-limiting steps (Fell, 1997). In the glycolytic pathway three reactions have large equilibrium constants (Reich & Sel'kov, 1981): hexokinase (HK), phosphofructokinase (PFK) and pyruvate kinase (PK). They are far from equilibrium *in vivo* (*e.g.* Mashego *et al.*, 2006; Teusink *et al.*, 2000). Displacement from equilibrium was thought to be a diagnose of control points in metabolic pathways. Indeed, it has been shown that near equilibrium reactions have little control on fluxes. Near equilibrium, the ξ term in Eq. (1.35) is very small, close to zero, and therefore, changes in f or θ have little effect on the rate (Heinrich & Rapoport, 1973). Moreover, the thermodynamic component $\frac{\mathrm{d}\ln\xi}{\mathrm{d}\ln x_i}$, of the elasticities towards substrates and products [Eq. (1.36)] becomes extremely high near the equilibrium, which means that the rate of catalysis is extremely sensitive to the concentrations of its susbtrates and products and is de-

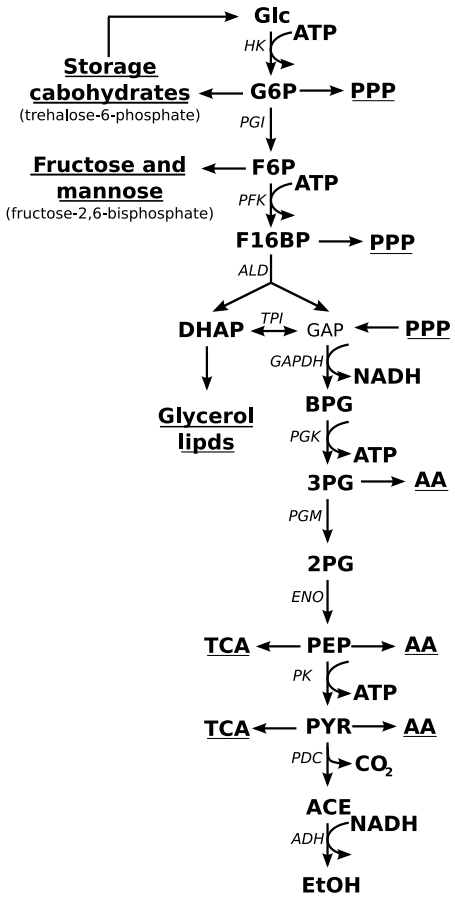

Figure 1.5: The glycolytic pathway. Metabolites are shown in bold, enzymes in italics and branching pathways are underlined. Abbreviations: i) enzymes: HK (hexokinase), PGI (phosphoglucose isomerase), PFK (phosphofructokinase), ALD (aldolase), TPI (triosephosphate isomerase), GAPDH (glyceraldehyde-3-phosphate dehydrogenase), PGK (phosphoglycerate kinase), PGM (phosphoglycerate mutase), ENO (enolase), PK (pyruvate kinase), PDC (pyruvate decarboxylase), ADH (alcohol dehydrogenase). ii) metabolites: Glc (glucose), G6P (glucose-6-phosphate), F6P (fructose-6-phosphate), F16P (fructose-1,6-bisphosphate), DHAP (dihydroxyacetone phosphate), GAP (glyceraldehyde-3-phosphate), BPG (1,3-bisphosphoglycerate), 3PG (3-phosphoglycerate), 2PG (2-phosphoglycerate), PEP (phosphoenolpyruvate), PYR (pyruvate), ACE (acetaldehyde), EtOH (ethanol). iii) pathways: PPP (pentose phosphate pathway), TCA (tricarboxylic acid pathway), AA (aminoacid synthesis pathways). Note that trehalose-6-phosphate, the inhibitor of hexokinase is an intermediate in the storage carbohydrate branch. Similarly fructose-2,6-bisphosphate, an activator of phosphofructokinase is an intermediate in the fructose and mannose synthesis branch.

termined by their ratio. However, the statement that enzymes far from equilibrium have high flux control is not true in general (Fell, 1997). The control of a metabolic step depends not only upon the thermodynamics of the reaction but also upon the kinetic properties of the catalyzing enzyme (Reich & Sel'kov, 1981; Hofmeyr, 1995). The rational engineering of metabolic fluxes requires, in addition to knowledge of the thermodynamics of the reactions, the investigation of the kinetic properties of its enzymes. Interactions between enzymes through metabolites are not limited to substrates and products but may also include inhibitors and allosteric modulators. In yeast glycolysis, the kinetic behavior of three enzymes has been reported to be affected by metabolites other than their substrates and products: HK, PFK and PK. Note that these enzymes happen to catalyse the reactions that are far from equilibrium.

Hexokinase phosphorylates glucose and produces glucose-6-phosphate with the phosphate deriving from ATP. In yeast, three isoenzymes can catalyse the hexokinase reaction: hexokinase I and II and glucokinase. Hexokinase I and II have been reported to be competitively inhibited *in vitro* by trehalose-6-phosphate (Blazquez *et al.*, 1993). The reported inhibition constant for trehalose-6-phosphrate was 0.04 mM for hexokinase II and 0.2 mM for hexokinase I. Glucokinase appears not to be inhibited by trehalose-6-phosphate. PFK is a multimodulated enzyme (Sols, 1981). In yeast it is activated by AMP (Ramaiah, 1974), ammonium ions (Sols & Salas, 1966), phosphate (Banuelos *et al.*, 1977) and fructose-2,6-bisphosphate (Bartrons *et al.*, 1982). PFK has long been thought of as the rate-limiting step of glycolysis (*cf.* Fell, 1997). However, its overproduction did not change the fermentation flux (Heinisch, 1986; Schaaff *et al.*, 1989). Davies and Brindle showed that overexpression of PFK was accompanied by a decrease in the concentration of its activator fructose-2,6-bisphosphate, which compensated for the increased concentration of the enzyme (Davies & Brindle, 1992). This is beautiful example of a mechanism through which an enzyme catalysing a reaction far from equilibrium looses flux control due to its kinetic properties. Pyruvate kinase catalyses the hydrolysis of phosphoenol pyruvate into pyruvate coupled to the phosphorylation of ADP. PK is strongly activated allosterically by fructose-1,6-bisphosphate, the product of PFK (Murcott *et al.*, 1992).

Kinetic regulation of enzymes by metabolites other than their substrates and products introduces an additional layer of kinetic regulation of a pathway. Rational approaches to flux engineering must take these interactions into account. Figure 1.5 also shows two other important aspects of the glycolytic and fermentative pathways: six of its reactions involve coenzymes (ATP and NADH) and seven of the thirteen metabolic intermediates (excluding coenzymes) are branching points, *i.e.* they participate in reactions outside glycolysis. Therefore, the glycolytic pathway is not an independent metabolic unit. The reactions that it comprises are embedded in a large and highly connected network.

1.4.2 Kinetic models of glycolysis

A number of kinetic computer models of the glycolytic pathway of *S. cerevisiae* have been constructed over the last 30 years. The earliest of these modeling efforts were concerned with the stability of steady states and in particular with the stoichiometric and regulatory structures of the glycolytic pathway to which the experimentally

observed phenomenon of sustained oscillations in yeast cultures and extracts could be ascribed (Betz & Chance, 1965; Boiteux *et al.*, 1975; Boiteux & Busse, 1989; Hess & Boiteux, 1968; Richter *et al.*, 1975) (see Section 1.2.1). The development of MCA stimulated the construction of kinetic models to investigate the distribution of flux control in glycolysis with the aim of amplifying or redirecting its flux (Aon & Cortassa, 1994; Delgado *et al.*, 1993; Galazzo & Bailey, 1990; Schlosser *et al.*, 1994). The more recent models of yeast glycolysis are detailed kinetic models based on *in vitro* determined kinetic equations (Rizzi *et al.*, 1997; Teusink *et al.*, 2000; Hynne *et al.*, 2001). Each of these detailed kinetic models was constructed with a different aim.

Rizzi *et al.* constructed a detailed kinetic model of yeast glycolysis based on published kinetic mechanisms and affinity constants. Dynamic experiments were used as input to fit the enzyme capacities in an attempt to determine these parameters *in vivo* instead of relying on *in vitro* determinations (Rizzi *et al.*, 1997). Teusink *et al* also constructed a detailed model, which was similar to that of Rizzi *et al.* Their objective, however, was not to fit parameters from *in vivo* data but to investigate the extent to which biochemical knowledge from *in vitro* studies could be used to predict the glycolytic flux and the concentrations of glycolytic intermediates (Teusink *et al.*, 2000). Hynne *et al.* constructed a detailed kinetic model with yet another aim, *i.e.* to use the dynamic characteristics of oscillating yeast cultures to estimate the parameters of the detailed kinetic model. Like Rizzi *et al.*, Hynne *et al.* aimed at *in vivo* parameter estimation. The approach of Hynne *et al.*, however, allowed the estimation of not only the enzyme capacities but also of the affinity constants (Hynne *et al.*, 2001).

These kinetic models share the unfortunate feature that their predictions are very poor for conditions that are different from those used in their construction. In contrast to phenomenological models, detailed models aim at the prediction of metabolic system properties (fluxes and metabolite concentrations) based on the characteristic of the components of the system (enzyme amounts, specificities and kinetics). The current paradigm is that knowledge of the properties of the parts should enable the prediction of the properties of the system. The construction of detailed kinetic models faces difficult challenges. First, the catalogue of enzyme properties of any real metabolic system is, at the time being, incomplete. Second, there are uncertainties on the available kinetic mechanisms and parameters and in particular upon the validity of these *in vitro* determined properties to predict the behavior of the enzymes *in vivo*. But perhaps the greatest challenge derives from the fact that living cells adapt to environmental changes by modulating the kinetic properties of their enzymes. Therefore, the kinetic parameters mesured in a given condition and used to construct a detailed kinetic model are likely to differ from those when the cells have adapted to a another, different condition.

Although detailed kinetic models should in principle provide a way of predicting the properties and behavior of metabolic systems, the demands on their construction are enormous. The performance of detailed kinetic models discussed above indicate that the available information upon enzyme properties is insuffcient or inadequate for the quantitative prediction of fluxes and metabolite concentrations even for yeast glycolysis, which is, perhaps, the most extensively studied pathway today.

1.4.3 Mechanisms of enzyme activity modulation

In the section "Regulation of Metabolic Networks" (Section 1.3), the concept of regulation was introduced and methods to quantify the local regulation enzyme rates were described. Enzyme rates depend upon the catalytic capacity of enzymes (V_{max}) and upon the interaction of these enzymes with the rest of metabolism. In this section, attention is given to the processes involved in the modulation of enzyme activities. At the same time, the section introduces an important and extensively studied adaptation to an external perturbation known as catabolite repression.

Cultures of *S. cerevisiae* that are glucose-limited, in stationary phase or growing on non-fermentable carbon sources are characterized by a fully oxidative metabolism of carbohydrates. Upon transfer of such cultures to glucose-rich conditions, a metabolic shift towards profuse alcoholic fermentation is observed. This phenomenon is caused by a number of mechanisms involving sensing of the external glucose concentration, signaling and modulation of enzyme activities in different ways. Together, these mechanisms are associated with the term catabolite repression (for reviews *cf.* Rolland *et al.*, 2002; Gancedo, 1998). Often the terms *repressed* and *derepressed* are used to refer to cultures with or without catabolite repression, respectively.

Covalent modification of enzymes

Allosteric modulations and covalent modifications are among the fastest enzyme-activity modulation processes (Fell, 1997). In Regulation Analysis as it was introduced above, allosteric modulations would be classified as a part of the metabolic regulation, while covalent modifications that affect V_{max}, would be scored as hierarchical regulation. The latter type of regulation is mediated by signal transduction pathways and often results in phosphorylation of the target enzyme.

The Ras-cAMP signaling pathway. A major glucose-signaling pathway involved in post-translational regulation of enzymes involved in carbon metabolism is the Ras-cAMP pathway (Rolland *et al.*, 2002). cAMP is synthesized from ATP in a reaction catalyzed by adenylate cyclase. cAMP then activates cAMP-dependent protein kinase A (PKA). Transfer of derepressed cells to sugar excess triggers a rapid, transient increase in cAMP, which initiates a phosphorylation cascade. It appears that the pathway plays a role in regulating the transition from the derepressed to the repressed state by means of transient PKA dependent phosphorylation of metabolic enzymes (Rolland *et al.*, 2002). This is consistent with the observation that PKA activity does not correlate with *steady-state* levels of cAMP and with the fact that glucose-induced activation of adenylate cyclase is followed by the repression of its expression (Rolland *et al.*, 2002).

Among the glycolytic enzymes, pyruvate kinase has been reported to be activated through PKA dependent phosphorylation (Portela *et al.*, 2002). Hexokinase I and II have also been reported to be phosphorylated *in vivo* but apparently not by PKA. The kinase responsible for the latter modification has not yet been identified (Kriegel *et al.*, 1994; Vojtek & Fraenkel, 1990).

Control of gene transcription

On longer time scales (minutes to hours) enzyme capacities are regulated by changing the concentrations of enzymes via changes in gene expression and protein degradation. The first process in the gene-expression cascade, transcription, has been studied extensively.

Transfer of derepressed yeast cultures to glucose rich conditions results in the repression of the transcription of many genes and the induction of others. Glucose-repressed genes include those encoding proteins involved in respiration (tricarboxylic acid cycle and electron-transport system), gluconeogenesis, the glyoxylate cycle, the uptake of alternative carbon sources, the high-affinity glucose transporters and a large group of stress response element (STRE)–controlled genes. The pathways involved in the regulation of these genes are the main glucose repression pathway and the so-called glucose induction pathway (Rolland *et al.*, 2002).

The main glucose-repression pathway. The main glucose-repression pathway is involved in the regulation of genes encoding proteins involved in respiration, gluconeogenesis, the glyoxylate cycle, the uptake of alternative carbon sources and the glucose transporters. The central components of this pathway are the Mig1 transcriptional repressor complex, the Snf1-protein kinase complex and protein phosphatase 1 (Rolland *et al.*, 2002).

Mig1 is a transcription regulator that binds to the promoters of glucose-repressible genes. Its function is dependent on its intracellular localization, which is regulated by phosphorylation (DeVit & Johnston, 1999; Ostling & Ronne, 1998). The Snf1-protein kinase is thought to be responsible for the phosphorylation of Mig1 (DeVit & Johnston, 1999; Ostling & Ronne, 1998; Treitel *et al.*, 1998) thereby causing its translocation from the nucleus to the cytoplasm. Snf1 activity is in turn inhibited by glucose (Wilson *et al.*, 1996; Woods *et al.*, 1994). Consequently genes, of which the expression is inhibited by Mig1, are repressed in glucose-excess conditions. The sensitivity of Snf1 to glucose inhibition is, however, modulated by its phosphorylation by a yet unidentified kinase (Wilson *et al.*, 1996; Woods *et al.*, 1994; Ludin *et al.*, 1998; Estruch *et al.*, 1992). Protein phosphatase 1 acts antagonistically to Snf1 in glucose repression, presumably by dephosphorylating Snf1 and thereby favoring its inhibition by glucose (Sanz *et al.*, 2000a,b; Ludin *et al.*, 1998). It is, however, unclear whether the protein phosphatase or the unidentified kinase are regulated by glucose and diminish or enforce the inhibition of Snf1 by glucose (Rolland *et al.*, 2002).

The gene *HXK2*, which encodes the glycolytic enzyme hexokinase II, has been shown to be involved in glucose repression (Zimmermann & Scheel, 1977; Entian & Zimmermann, 1980; Michels & Romanowski, 1980). Deletion of the gene *HXK2* results in alleviation of glucose repression (Diderich *et al.*, 2001; Raamsdonk *et al.*, 2001; Petit *et al.*, 2000) but the mechanisms through which hexokinase II triggers glucose repression is not fully understood. Although phosphorylating capacity (*i.e.* the summed capacity of the hexose phosphorilating enzymes) correlates with glucose repression (Ma *et al.*, 1989; Rose *et al.*, 1991), it is not the sole determinant of glucose repression (Rose *et al.*, 1991). It has been shown that Hexokinase II resides partly in the nucleus (Randez-Gil *et al.*, 1998) and that this nuclear localization depends

upon Mig1 (Ahuatzi *et al.*, 2004). The role of hexokinase II in glucose repression and glycolytic fluxes in starved and unstarved yeast cells is discussed in Chapter 5.

Induction of glycoytic genes. Glucose repression is accompanied by an increase in the glycolytic capacity. The latter is ascribed to the induction of glycolytic genes and the regulation of glucose transporters. This induction is thought to be triggered partially by a transient increase of the concentrations of some glycolytic intermediates (Boles *et al.*, 1993), but how these metabolic signals are transmitted is unclear. It has been shown that the GCR1/GCR2/RAP1 system is involved in the global regulation of the transcription of glycolytic genes. This complex is a *trans*-acting positive regulator of transcription that binds to a motif which is conserved in most glycolytic genes (Clifton & Fraenkel, 1981; Uemura *et al.*, 1997).

Transcription of some glycolytic genes, *i.e.* PGK (phosphoglycerate kinase), ENO2 (enolase), PYK1 (pyruvate kinase), PDC1 (pyruvate decarboxylase) and ADH1 (alcohol dehydrogenase), has been shown to be controlled by the GCR1/GCR2/RAP1 system and to be induced up to 20-fold in the presence of fermentable sugars (Chambers *et al.*, 1995).

Regulation of the expression of hexose transporters. *S. cerevisiae* has 18 hexose transporter homologues displaying different substrate affinities (Hxt1-17 and Gal2). Gal2 is a galactose transporter that also transports glucose (Boles & Hollenberg, 1997; Kruckeberg, 1996). Rgt3 and Snf3 are also homologues, but they have been shown not to participate directly in glucose transport but rather to act as glucose sensors (Reifenberger *et al.*, 1995; Lang & Cirillo, 1987). Snf3 is required for the induction of transcription of HXT2, HXT3 and HXT4 by low glucose levels (Skowyra *et al.*, 1997) while Rgt2 is responsible to the constitutive *i.e.* glucose-independent expression of HXT1 and the full induction of the latter by high glucose concentrations (Ozcan *et al.*, 1998). The sensing mechanism and the signalling routes of Snf3 and Rgt2 have not been completely elucidated (but see Ozcan & Johnston, 1999).

Studies upon the regulation of hexose transporters distinguish three conditions: absence of glucose (*e.g.* during growth on non-fermentable sugars), low, and high glucose concentrations. In the absence of glucose, the expression of low and intermediate affinity transporters (Hxt1-4) is repressed by Rgt1. Rgt1 is a zinc-finger-containing DNA-binding protein that binds to and inactivates the promoters of several genes (Ozcan *et al.*, 1996). In the presence of glucose, the repression function of Rgt1 is repressed by Snf3, leading to de-repression of low and intermediate affinity hexose transporters (Ozcan *et al.*, 1996). At high glucose concentrations, the expression of high affinity transporters (Hxt6 and Hxt7) is repressed by the main glucose repression pathway through Mig1 and the expression of Hxt1 is induced by Rgt2. These regulations result in the expression of: the high-affinity transporters (Hxt6 and Hxt7) in the absence or at low concentrations of glucose, the expression of intermediate-affinity transporters (Hxt2 and Hxt4) at low glucose concentrations but not in its absence or excess, and additional, full induction of the constitutively expressed low affinity transporter Hxt1 under glucose excess.

Posttranscriptional regulation

The regulation of transcription has received substantial attention in the last decades. However, it is becoming more and more evident that transcription is an important but not unique determinant of protein levels. The processes of mRNA decay, translation and protein degradation are likely to play an important role in the regulation of protein concentrations (Vilela & McCarthy, 2003; Smirnova *et al.*, 2005; Tucker & Parker, 2000; Wilusz & Wilusz, 2004, see also Chapter 4).

Little is known about the translational regulation of the glycolytic and fermentative proteins. One way of regulating translation in *S. cerevisiae* is via upstream open-reading frames (uORFs) that can partly or completely disable the initiation of the translation machinery (McCarthy, 1998). uORFs are probably restricted to a small number of mRNAs that are expressed at low levels and encode regulatory proteins (Vilela & McCarthy, 2003). This type of regulation has, however, also been reported for the enzyme carbamoyl phosphate synthetase in *S. cerevisiae* (Gaba *et al.*, 2001). Regulation of glycolytic proteins at the level of translation has not yet been reported. Recently, however, the mRNA translation status (bound or unbound to ribosomes) has been measured genome-wide (Arava *et al.*, 2003). Hopefully, more insights in the translational regulation will be gained in the near future.

Even less is known about degradation of glycolytic enzymes. Although targeted degradation of enolase 2 has already been reported (Larsen *et al.*, 2001), regulatory mechanisms involved in the targeted degradation of glycolytic enzymes have not been systematically investigated. As in the case of translation, recent developments have achieved high-throughput measurements of protein turnovers (Pratt *et al.*, 2002) and new insights into protein degradation may become available in the near future.

The high-affinity glucose transporters *HXT6* and *HXT7* have been shown to be rapidly degraded during nitrogen starvation in the presence of high concentrations of fermentable sugars. Under these conditions, the degradation of these high-affinity glucose transporters takes place in the vacoule and is preceeded by their internalization by endocytosis and the delivery of the endosome's content to the vacoule (Krampe & Boles, 2002)

1.4.4 The bakers' yeast industry

The bakers' yeast industry aims at producing high quality yeast at a low price. Fermentative capacity is a particularly important quality parameter. It is defined as the rate of CO_2 production immediately upon transfer to the bread dough. The dough environment is considered to be anaerobic, saturated with CO_2 and containing an excess amount of fermentable sugars like glucose, fructose, maltose and sucrose (van Hoek, 2000). In the laboratory, dough conditions are mimicked in anaerobic vessels to which an excess of sugar is added. Fermentative capacity is then measured by monitoring the concentration of CO_2 or ethanol over a period of 0.5 to 1 hour (van Hoek *et al.*, 1998b).

Commercial production of bakers' yeast is performed in large reactors ($> 100m^3$). Molasses is used as the main source of carbon and free energy and ammonia or urea are used as nitrogen source. The final stage of the production is a "maturation"

period aimed at improving the storage stability through accumulation of storage carbohydrates induced by nitrogen deprivation. The production process yields a broth containing between 40 and 80 grams of dry biomass per liter (van Hoek, 2000).

The cultivation conditions determine the quality parameters of the bakers' yeast. Often, different quality parameters and the cost effectiveness of the production process cannot be optimized simultaneously and compromises are unavoidable. For instance, production at high growth rates leads to high fermentative capacities, but also results in a low biomass yield and a poor storage stability (Burrows, 1979; van Hoek *et al.*, 1998b).

Nutrient starvation

Starvation for nutrients is perhaps one of the most common stress conditions experienced by microorganisms in their natural habitat and may affect them most of their life span. Nutrient starvation is also a relevant phenomenon in the bakers' yeast industry. At the final stages of the production and during storage the cells are starved. Starvation affects several quality parameters, of which the most important are the storage stability and the fermentative capacity. Nutrient starvation has been reported to decrease the fermentative capacity (Nilsson *et al.*, 2001b; Rossell *et al.*, 2002; Thomsson *et al.*, 2003). The severity of the reduction depends upon the type of starvation as well as upon the initial physiological state of the cells (Nilsson *et al.*, 2001b). Fermentative capacity changes in response to nutrient starvation are accompanied by a myriad of changes in enzyme levels that differ in magnitude and direction (Nilsson *et al.*, 2001b; Rossell *et al.*, 2002; Thomsson *et al.*, 2003). The precise mechanism by which these changes are brought about is not known. It has been reported that during nitrogen starvation non-specific protein degradation via autophagy is enhanced (Abeliovich & Klionsky, 2001). The process of nutrient depletion and adaptation to the exhausted medium occurs in phases, each marked by characteristic gene expression changes. Gene expression has been measured in batch cultures that were monitored beyond the point of carbon depletion. These experiments indicated that at the diauxic shift (*i.e.* the shift from respiro-fermentative catabolism of glucose to the respirative catabolism of ethanol upon glucose exhaution) cells induce the expression of genes involved in respiration, fatty acid metabolism and the glyoxylate cycle. These changes persist through the post-diauxic phase, but many subside slightly as the cells enter the stationary phase. In the late stationary phase the expression of many genes is decreased, including that of genes involved in secretion, membrane and cell wall synthesis, amino acid metabolism, cell-cycle progression and other process required for growth and division. For a review of the transcriptome responses to nutrient starvation see citetGasch02. However, the interpretation of these transcriptome analyses in the context of changes of fermentative capacity is hindered by the differences between starvation protocols in different studies. While in most studies of the effect of nutrient starvation on fermentative capacity the cells were deprived suddenly of a certain nutrient, the gene-expression studies were performed on cultures in which the nutrient was depleted gradually by consumption.

1.5 Outline of the Thesis

The study of metabolic regulation is not underpinned by a theory explaining how cells will respond to a given stimulus. Such a theory will have to be built by induction based on a body of observations. In this thesis, Regulation Analysis is used to organize experimental observations in an unambiguous and quantitative manner. Not only is the method applied to the analysis of experimental observations, also its scope and biochemical interpretations is clarified. An effort made to extend the scope of Regulation Analysis to the dissection of the regulation of enzyme capacities by the different processes in the gene-expression cascade, from transcription to catalytic activity. Further, with the benefit of the unambiguous description, the biological significance of our experimental findings is discussed from a novel point of view.

In Chapter 2, Regulation Analysis is expounded in terms of the biochemical interpretation of the possible numerical outcomes of the regulation coefficients. The method is then applied to quantify the metabolic and hierarchical regulation of glucose uptake upon nitrogen or carbon starvation of *S. cerevisiae*. This is the first instance of unambiguous and quantitative statements upon the regulation of glucose uptake.

Further, experiments revealed that the affinity of glucose transport was modulated during starvation, presumably through changes in the isoenzyme distribution of the transporters (*cf.* also Diderich *et al.*, 1999a). This feature of biological complexity required a refinement of the interpretation of Regulation Analysis.

In Chapter 3 two aspects of the regulation of metabolic fluxes are distinguished: (i) the local regulation of the fluxes through the individual steps of the pathway and (ii) the regulation of the global flux through the pathway. Regulation analysis quantifies the former. In this chapter the local regulation of the fluxes through individual enzymes of glycolysis and alcoholic fermentation is quantified for the adaptation of *S. cerevisiae* to nitrogen or carbon starvation. This description is used to evaluate *experimentally* the predictions of three hypotheses proposed for the global regulation of fluxes: metabolic, single- and multi-site modulation.

In Chapter 4 the hierarchical regulation of local fluxes is further dissected into contributions by the various processes involved in the gene-expression cascade. The V_{max} changes that constitute the hierarchical component of the regulation, are brought about by a number of processes that potentially include the regulation of transcription, mRNA decay, translation, protein degradation and posttranslational modifications. This chapter describes the refinement of Regulation Analysis aiming at the dissection of the contributions of all these processes to the regulation of enzyme capacities. The method is applied to dissect the regulation of fluxes through the glycolytic enzymes when *S. cerevisiae* is challenged by the absence of oxygen and the presence of the uncoupler benzoic acid.

Chapter 5 describes the regulation of glycolytic and fermentative fluxes in response to the deletion of the *HXK2* gene encoding hexokinase II. The regulation of fluxes is described from two different points of view. First, Regulation Analysis is applied to dissect the contributions of hierarchical and metabolic regulation. Experiments and analysis evidence the pleiotropy of the *HXK2* gene at the level of active enzyme concentrations and local fluxes. Second, the effect of the deletion of *HXK2* is described

in terms of its effects on fermentative capacity and its response to nutrient starvation. These findings and others reported in the literature suggest an inverse correlation between glucose repression and the resilience of fermentative capacity towards nutrient starvation.

Finally, Chapter 6 contains a general discussion of the methodologies, experiments and conclusions reported in this thesis.

Chapter 2

The Regulation of Glucose Influx

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.

2.1 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 (Daran-Lapujade *et al.*, 2004; ter Kuile & Westerhoff, 2001).

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

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array technology (Castrillo & Oliver, 2004). 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 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 (Savageau, 1969a,b; Heinrich & Rapoport, 1973; Kacser & Burns, 1973). 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 cellular system, but its outcomes are often accessible to experimental determination. Ter Kuile and Westerhoff proposed a method called "Regulation analysis", in order to disentangle quantitatively "hierarchical" from "metabolic" regulation of flux (Westerhoff *et al.*, 2000; ter Kuile & Westerhoff, 2001). 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 dissect transcriptional from translational control. Although Regulation Analysis is compatible with and complementary to Metabolic Control Analysis (Heinrich & Rapoport, 1973; Kacser & Burns, 1973) and its extension Hierarchical Control Analysis (Kahn & Westerhoff, 1991), 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:

$$v = v(e, \mathbf{x}, \mathbf{k}) = f(e) \cdot g(\mathbf{x}, \mathbf{k}) \tag{2.1}$$

in which v is the rate,e is the concentration of the enzyme, $\mathbf{x}$ is the vector of substrate, product and other effector concentrations and $\mathbf{k}$ is a vector of constants parametrizing the strength with which the enzymes interact with their substrates, products and allosteric effectors. 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:

$$\ln v = \ln f(e) + \ln g(\mathbf{x}, \mathbf{k}) \tag{2.2}$$

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 $g(\mathbf{x}, \mathbf{k})$, it is useful to divide as follows:

$$1 = \frac{\Delta \ln v}{\Delta \ln J} = \frac{\Delta[\ln f(e) + \ln g(\mathbf{x}, \mathbf{k})]}{\Delta \ln J} = \frac{\Delta \ln f(e)}{\Delta \ln J} + \frac{\Delta \ln g(\mathbf{x}, \mathbf{k})}{\Delta \ln J} = \rho_h + \rho_m \quad (2.3)$$

in which ρ_h is the hierarchical regulation coefficient and ρ_h 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:

$$\rho_h = \frac{\Delta \ln V_{max}}{\Delta \ln J} \quad (2.4)$$

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 (Christensen & Nielsen, 2000; Stephanopoulos, 1999). 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 the discussion section 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 (Nilsson *et al.*, 2001b; Rossell *et al.*, 2002; Thomsson *et al.*, 2003). Also it is known that proteins are actively degraded under starvation conditions (Abeliovich & Klionsky, 2001), among which glucose transporters are no exception (Krampe & Boles, 2002).

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 rate equation for a symmetric carrier (Stein, 1986).

$$v = V_{max} \frac{\left(\frac{[Glc]_{out}}{K_{m,out}} - \frac{[Glc]_{in}}{K_{m,in}} \right)}{1 + \frac{[Glc]_{out}}{K_{m,out}} + \frac{[Glc]_{in}}{K_{m,in}} + \alpha \cdot \frac{[Glc]_{out}}{K_{m,out}} \cdot \frac{[Glc]_{in}}{K_{m,in}}} \quad (2.5)$$

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 Eq. (2.5) in Eq. (2.3) gives:

$$\frac{\Delta \ln v}{\Delta \ln J} = \frac{\Delta \ln V_{max}}{\Delta \ln J} + \frac{\Delta \ln g([Glc]_{out}, [Glc]_{in}, K_{m,out}, K_{m,in}, \alpha)}{\Delta \ln J} = \rho_h + \rho_m = 1 \quad (2.6)$$

This equation emphasizes on 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 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.

2.2 Materials and Methods

2.2.1 Growth and starvations

Saccharomyces cerevisiae strain CEN-PK 113-7D (*MATa MAL2-8^c SUC2*) was grown in controlled batch cultures of 1.5 liters at a stirrer speed of 800 rpm and at 30°C in defined mineral medium containing 101 mM glucose (Verduyn *et al.*, 1992). 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_{600nm} 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 (approximately 1g dry weight l^{-1}) at pH 6.0. The suspensions, of approximately 300 ml, were kept in 2-liter shake flasks on a rotary shaker at 30°C and 200 rpm without pH control for 24 hours. To avoid dual starvation, the growth medium lacking ammonium contained 177 mM glucose. After 24 hours the glucose concentration in the supernatant of nitrogen-starved culture 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 hours 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

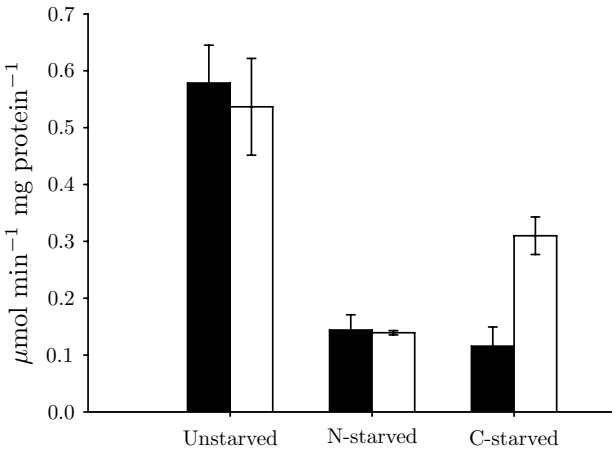

Figure 2.1: Comparison of steady-state glucose consumption flux with the capacity of the glucose transporters at 0.1 M as determined by zero-*trans* influx experiments. Steady-state glucose consumption flux (black bars) and zero-*trans* influx glucose transport capacity (white bars). The error bars represent the standard deviation of two independent experiments carried out with different batches of cells.

density of 6% wet weight (10 and 5 g dry weight l^{-1} respectively) and kept on ice for at most 1 hour. Similarly, for the measurement of zero-*trans* influx of glucose, cells were harvested by centrifugation and resuspended in growth medium without carbon nor 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) and kept on ice for at most 1 hour.

2.2.2 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.* for determination of the fermentative capacity (van Hoek *et al.*, 1998b), 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 (300mm x 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})

2.2.3 Zero-*trans* influx of glucose

Zero-*trans* influx of ^{14}C radiolabelled glucose was determined in a 5 second uptake assay at 30 C according to Walsh *et al.* (1994), 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 minutes 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 regressing using

Table 2.1: Kinetic parameters of the irreversible Michaelis-Menten equations after fitting to the dependence of rate on glucose concentration (*cf.* Figure 2.2). 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.

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

SigmaPlot 2001 version 7.0 (SPSS Inc.).

2.3 Results

2.3.1 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 minutes. 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 hours 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 2.1 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 (Figure 2.1). 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 minutes) in any of the conditions (not shown).

2.3.2 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

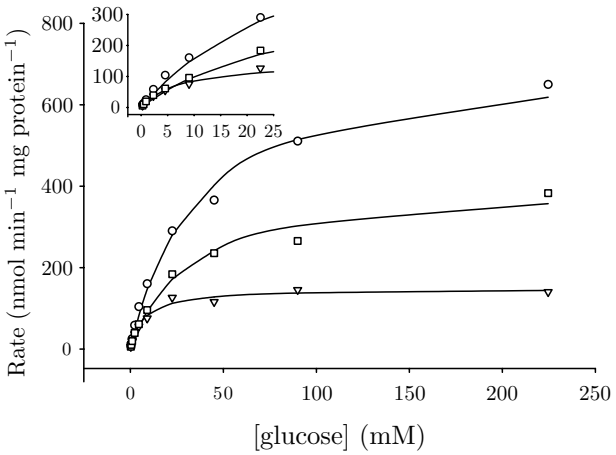

Figure 2.2: Dependence of glucose uptake rate upon glucose concentration as measured in zero-*trans* influx experiments. The rate of glucose uptake at different glucose concentrations was measured for three conditions: unstarved (circles), nitrogen starved (triangles) and carbon starved (squares). Symbols 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 2.1). The inset illustrates the rate dependence upon glucose concentration at the lower glucose concentrations.

transporters. To quantify the zero-*trans* uptake kinetics of glucose, the uptake of radio-labeled glucose was measured during 5 seconds 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 (Figure 2.2). During N-starvation a decrease in K_m was observed, while the affinity during carbon starvation was unchanged within statistical error (Table 2.1).

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, Figure 2.2). 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 (Teusink *et al.*, 1998) was negligible, *i.e.* internal glucose was and remained low. In contrast, carbon-starved cells possessed a 3- to 4- 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 (Stein, 1986, Eq. (2.5)) with the parameters reported for carbon starved cells (Table 2.1) and using

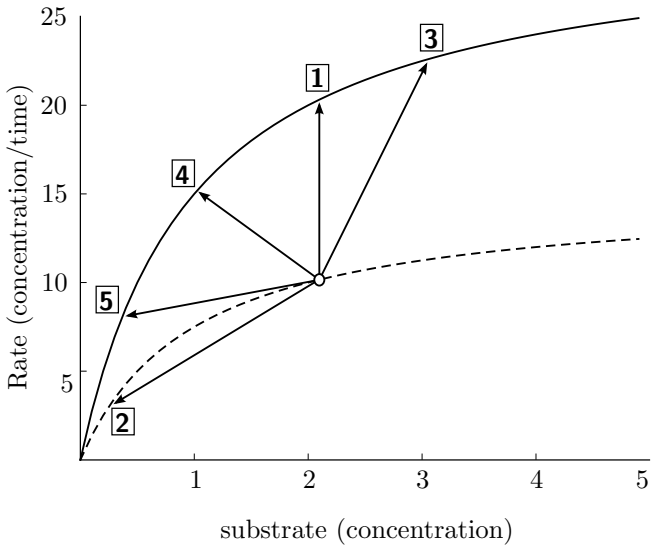

Figure 2.3: Biochemical interpretations of regulation coefficients: the dependence on the substrate concentration is depicted for two enzymes described by irreversible Michaelis-Menten kinetics. Both enzymes have a K_m of 1 (concentration units). They differ in their V_{max}-values (concentration/time 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.

an estimated cell volume of 3.75 μl per mg protein (de Koning & van Dam, 1992). 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.* (1996), 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.

2.3.3 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. Figure 2.3 illustrates the biochemical interpretation of each of these five categories. In this figure (Figure 2.3), 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):

- $\boldsymbol{\rho_h = 1}$ means that the relative change of V_{max} equals that of the flux. This implies that there is no metabolic regulation. In the example on Figure 2.3 this is obvious from the fact that the substrate concentration does not change (see arrow 1, Figure 2.3). In more complex 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.

- $\boldsymbol{\rho_h = 0}$ 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.

- $\boldsymbol{0 < \rho_h < 1}$ means that the relative increase in V_{max} is smaller than the relative increase in flux. From the summation theorem, Eq. (2.6) 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 (Figure 2.3).

- $\boldsymbol{\rho_h > 1}$ 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 in the product concentration will go up, making metabolic regulation buffer away the regulation through gene expression.

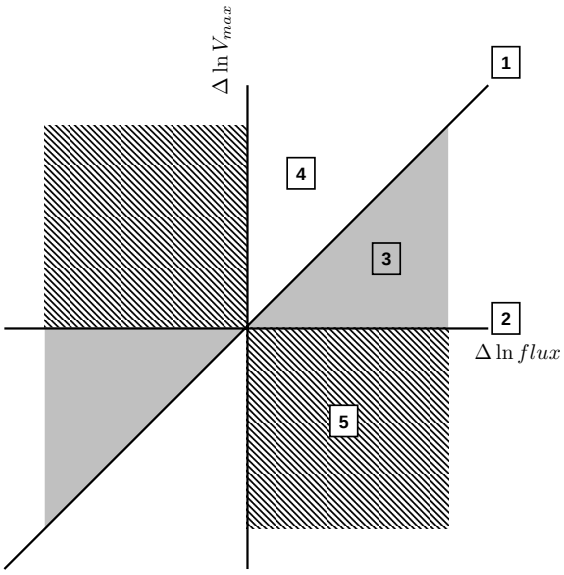

Figure 2.4: Graphical representation of the different modes of steady-state flux through an enzyme. This coordinate system with axes corresponding to the relative changes in flux and V_{max} allows a graphical representation of any process, in which these properties are changed, in a common plot. Furthermore, 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 boxed 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.

- $\boldsymbol{\rho_h < 0}$ 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 "$\Delta \ln V_{max}$ 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 Figure 2.4 correspond to the different categories distinguished above. This illustration (Figure 2.4) 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

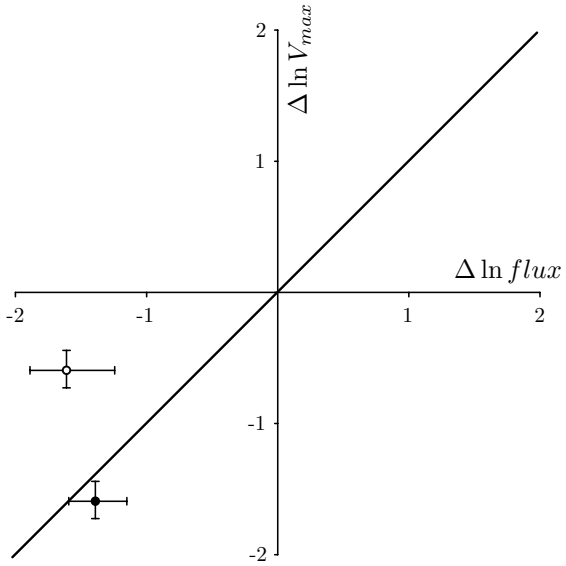

Figure 2.5: Regulation of glucose flux by glucose transport capacity upon starvation. A graphical representation of ρ_h in a coordinate system with "$\Delta \ln flux$" and "$\Delta \ln V_{max}$" as axes. Nitrogen (closed circle) and carbon (open circle) starvations are represented, error bars correspond 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 Figure 2.1 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).

relative change in the capacity of the glucose transporters, with a ρ_h of 1.1 ± 0.2 (dimensionless, error indicates standard deviation). This implies that regulation is classified best in category 1 (Figures 2.4 and 2.5, 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, Figures 2.4 and 2.5), 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.

2.4 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 (ter Kuile & Westerhoff, 2001; Westerhoff *et al.*, 2000). 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 (Rossell *et al.*, 2002; Thomsson *et al.*, 2003, Figure 2.1), 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" (Rossell *et al.*, 2002) or "the changes in flux were not reflected by the changes in glucose transport capacity" (Thomsson *et al.*, 2003). 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 (Westerhoff *et al.*, 2000).

We have 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 have found that both glucose consumption and transport capacity were decreased during starvation. However, the decrease of glucose transport relative to glucose flux was very different in the two types of starvation (Figure 2.1). 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.* the 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 minutes versus 5 seconds). 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 (Figure 2.2 and Table 2.1). Changes in the apparent affinity of transporters when cells are challenged by an environmental change are well known (Walsh *et al.*, 1994) and

they can be ascribed to changes in the isoenzyme distribution of the transporters (Diderich *et al.*, 1999a). *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 (Kruckeberg, 1996; Ozcan & Johnston, 1999; Boles & Hollenberg, 1997). 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 (Diderich *et al.*, 1999a).

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 (Introduction) 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. (2.1), 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. (2.5) and (2.6). 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 through *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. (2.1) becomes:

$$\begin{aligned} v &= v_1(e_1, \mathbf{x}) + v_2(e_2, \mathbf{x}) \\ &= f_1(e_1) \cdot g_1(\mathbf{x}) + f_2(e_2) \cdot g_2(\mathbf{x}) \end{aligned} \tag{2.7}$$

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

$$v = V'_{max} \cdot g'(\mathbf{x}, V_{max_1}/V_{max_2}) \tag{2.8}$$

with:

$$V'_{max} = V_{max_1} + V_{max_2}$$

and

$$\begin{aligned} g'\left(\mathbf{x}, \frac{V_{max_1}}{V_{max_2}}\right) &= \frac{V_{max_1}}{V_{max_1} + V_{max_2}} \cdot g_1(\mathbf{x}) + \frac{V_{max_2}}{V_{max_1} + V_{max_2}} \cdot g_1(\mathbf{x}) \\ &= \frac{\frac{V_{max_1}}{V_{max_2}}}{\frac{V_{max_1}}{V_{max_2}} + 1} \cdot g_1(\mathbf{x}) + \frac{1}{\frac{V_{max_1}}{V_{max_2}} + 1} \cdot g_2(\mathbf{x}) \end{aligned} \tag{2.9}$$

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 comprises 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.

Acknowledgements

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Chapter 3

The Regulation of Enzyme Rates and the Glycolytic Flux

An important question is to what extent metabolic fluxes are regulated by gene-expression or by metabolic regulation. There are two distinct aspects to this question: (i) the local regulation of the fluxes through the individual steps in the pathway, and (ii) the influence of such local regulation upon the pathway's flux. We developed Regulation Analysis so as to address the former aspect, for *all* steps in a pathway. We demonstrate the new method for the issue of how *Saccharomyces cerevisiae* regulates the fluxes through its individual glycolytic and fermentative enzymes when confronted with nutrient starvation. Regulation was dissected quantitatively into: (i) changes in maximum enzyme activity (V_{max} – called hierarchical regulation), and (ii) changes in the interaction of the enzyme with the rest of metabolism (called metabolic regulation). Within a single pathway, the regulation of the fluxes through individual steps varied from fully hierarchical to exclusively metabolic. Existing paradigms of flux regulation (such as single- and multi-site modulation, and exclusively metabolic regulation) were tested for the first time for a complete pathway, and falsified for a major pathway in an important model organism. We propose a subtler mechanism of flux regulation, with different roles for different enzymes, *i.e.* "leader", "follower" or "conservative", the latter attempting to hold back the change in flux. This study makes the subtlety, so typical for biological systems, tractable experimentally, and invites reformulation of the questions concerning the drives and constraints governing metabolic flux regulation.

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3.1 Introduction

The flux through a metabolic pathway is determined by the activities of its enzymes and by their interactions with other enzymes. Metabolic flux changes have often been observed in response to environmental or genetic changes. In the yeast *Saccharomyces cerevisiae*, for example, changes in glycolytic flux have frequently been found to be accompanied by a myriad of changes in glycolytic enzyme activities (Daran-Lapujade *et al.*, 2004; van Hoek *et al.*, 1998b, this work) or amounts (Nilsson *et al.*, 2001b), which varied in magnitude and direction. The complexity of interactions between enzymes translates into a vast possibility space of combinations of enzyme activity modulations leading to the same flux change. We wondered how the cell actually regulates its fluxes.

Among the proposed mechanisms for metabolic flux changes, the two clearest hypotheses are: (i) modulation of single rate-limiting enzymes, and (ii) multi-site modulation, *i.e.* simultaneous and proportional modulation of all enzymes in the pathway, thus causing a change in flux while leaving metabolite concentrations unchanged (Fell & Thomas, 1995). Although single rate-limiting enzymes exist, control of flux is quite often distributed over several enzymes (Fell, 1992). In the latter case, modulation of a single enzyme is likely to be an ineffective mechanism for changing a pathway's flux. Indeed, attempts to correlate flux changes with changes in single enzyme activities or levels have failed consistently (Daran-Lapujade *et al.*, 2004; Nilsson *et al.*, 2001b; van Hoek *et al.*, 1998b). In contrast, the opposing theory of multi-site modulation has met supporting examples, such as lipogenesis in mice, the urea cycle in rats, and photosynthesis in green plants (Fell & Thomas, 1995). It is not clear, however, how general this mechanism is, and whether indeed all enzyme activities changed in proportion to the flux.

An important question is to what extent metabolic fluxes are regulated by enzyme capacity V_{max} and to what extent by metabolic regulation. According to one paradigm, metabolic fluxes at steady-state are regulated through enzyme capacity changes (*e.g.* achieved through changes in gene expression). An orthogonal paradigm has metabolic regulation as dominant. Single- and multi-site modulations assume that flux changes are regulated through changes in the capacity of enzymes within the pathway, *e.g.* through transcription regulation and/or through covalent modification. The single-enzyme modulation hypothesis does not exclude the possibility of metabolic regulation, but it does assume a leading role of gene expression. In its strongest form, multi-site modulation, on the other hand, excludes the possibility of metabolic regulation, and proposes metabolite homeostasis as a constraint to regulatory processes. In fact, strong metabolite homeostasis of the glycolytic intermediate glucose-6-phosphate has been demonstrated in rat and human muscle during large changes in glucose consumption and the mechanism through which metabolite homeostasis was attained has been clearly elucidated (Shulman *et al.*, 1995).

The idea is as follows. Because enzymes are catalysts (and not substrates), enzyme rate equations are usually of the shape:

$$v = v\,(e, \mathbf{x}, \mathbf{k}) = f(e) \cdot g(\mathbf{x}, \mathbf{k}) \tag{3.1}$$

in which v is the rate, e is the concentration of enzyme, $\mathbf{x}$ is a vector of concentrations

of substrates, products and other metabolic effectors, and **k** is a vector of constants parametrizing the strength with which the enzymes interact with their substrates, products and allosteric effectors. The important characteristic of the above equation is that the multipliers are cross-independent. This means that f does not depend upon **x** and **k**, and g does not depend upon e. Exceptions to Eq. (3.1) exist, for instance in some cases of strong substrate channeling, and in this sense this is a first approach. $f(e)$ describes the dependency of the rate upon the enzyme concentration and can be taken to equal V_{max}, while $g(\mathbf{x}, \mathbf{k})$ describes the interaction of the enzyme with the rest of metabolism through metabolite concentrations and the corresponding affinity constants.

The dissection and quantification of f and g is achieved by translating Eq. (3.1) into logarithmic space, considering a change between two steady states, and dividing both sides of the equation by the relative change in steady-state flux J. Since at steady state the flux J equals the enzyme rate i v, this results in:

$$1 = \frac{\Delta \ln f(e)}{\Delta \ln J} + \frac{\Delta \ln g(\mathbf{x}, \mathbf{k})}{\Delta \ln J} = \rho_h + \rho_m \tag{3.2}$$

ρ_h is the "hierarchical regulation coefficient", quantifying the relative contribution of changes in active enzyme concentration to the regulation of the enzyme's flux. ρ_m is the "metabolic regulation coefficient", quantifying the relative contribution of changes in the interaction of the enzyme with the rest of metabolism to the regulation of the enzyme's flux. For a more elaborate description and discussion of the method see Chapter 2. The term "hierarchical regulation coefficient" was introduced by ter Kuile & Westerhoff (2001) because the V_{max} depends on the complete gene-expression cascade of transcription, translation, posttranslational modification, and mRNA and protein degradation. The two regulation coefficients sum up to one (summation theorem for the regulation of flux) implying that determination of one will yield the other automatically (ter Kuile & Westerhoff, 2001, Chapter 2). In practice the hierarchical regulation coefficient is more readily determined, since $f(e)$ usually can be taken to equal V_{max}, and the V_{max} as well as the flux J through the enzyme can be measured or estimated in most cases. Regulation Analysis introduces the possibility of making unambiguous and quantitative descriptions of the regulation of fluxes through individual enzymes embedded in biochemical networks of any complexity, in response to any number or kind of simultaneous perturbations.

In this study, Regulation Analysis is applied to the regulation of the flux through individual glycolytic and fermentative enzymes in *S. cerevisiae* during nutrient starvation. Starvation for nutrients is perhaps one of the most common stress conditions experienced by microorganisms in their natural habitat and it may affect most of the organisms' life span. Nutrient starvation is also relevant for the industrial production of baker's yeast. At the final stages of production and during storage cells are starved, and this affects several quality parameters among which the fermentative capacity (Nilsson *et al.*, 2001b; Rossell *et al.*, 2002) (the specific rate of CO_2 production under anaerobic conditions with excess of sugar, which almost equals the rate of ethanol formation (van Hoek *et al.*, 1998b)).

Using Regulation Analysis we here dissect quantitatively the regulation of fluxes through individual glycolytic and fermentative enzymes in response to nutrient star-

vation. Our experimental results served to test three regulatory paradigms (*i.e.* single enzyme, multi-site, all metabolic). The results evidence a more subtle regulation of cell function and show that the new method allows delineating *experimentally* the regulation of flux. Our results suggest that different enzymes in a common pathway play different roles in the regulation of the pathway's flux.

3.2 Materials and Methods

3.2.1 Growth and starvations

The growth and starvation procedures have been described in detail in Chapter 2. Briefly, *Saccharomyces cerevisiae* strain CEN-PK 113-7D (*MATa MAL2-8^c SUC2*) was grown in pH controlled batch cultures at 30°C in defined mineral medium containing 101 mM glucose (Verduyn *et al.*, 1992) kept at pH 5.0. Cells were harvested by centrifugation at an OD_{600nm} 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 the corresponding medium to a cell density of 0.75% wet weight (approximately 1g dry weight l^{-1}) at pH 6.0. The suspensions, of approximately 300 ml, were kept in 2-liter shake flasks on a rotary shaker at 30°C and 200 rpm without pH control for 24 hours. For the measurement of steady-state fluxes, the cells were harvested by centrifugation and resuspended in growth medium without a carbon source and kept on ice for at most 1 hour. Similarly, for the measurement of zero-*trans* influx of glucose, cells were harvested by centrifugation and resuspended in growth medium lacking carbon and nitrogen sources, and kept on ice for at most 1 hour.

3.2.2 Steady-state fluxes

Steady-state fluxes were measured for 30 minutes in a cell suspension kept anaerobic at 30°C in a setup described by van Hoek *et al.* (1998b) for the determination of fermentative capacity, with the modification that the headspace was flushed with N_2 instead of with CO_2. Ethanol, glucose, glycerol, succinate, acetate and trehalose were measured by HPLC (300 mm x 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}). Glycogen was assayed according to Parrou & Francois (1997). The rate of carbon dioxide production was calculated from the production rates of ethanol and acetate.

The flux through the glucose transporter (GLT) was taken as equal to the measured glucose consumption flux. The fluxes through enzymes downstream hexokinase (HK) were calculated from the steady-state rates of ethanol and glycerol production. Figure 3.1 shows a scheme of the pathway. Enzymes with the same flux are boxed together. The flux through HK, glucose-6-phosphate isomerase (PGI), 6-phosphofructokinase (PFK) and aldolase (ALD) was calculated by dividing the sum of the glycerol and ethanol fluxes by two. The flux through triose-phosphate isomerase (TPI) was calculated by subtracting the rate of glycerol from the flux through the previous block (HK until ALD), and the flux through the enzymes downstream

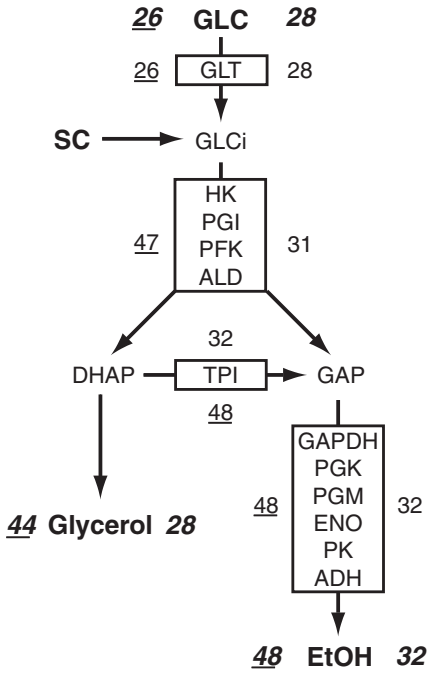

Figure 3.1: Schematic representation the glycolytic and fermentative pathways. In this simplified scheme of the glycolytic and fermentative pathways, enzymes are boxed and those with the same flux are boxed together. Measured fluxes are depicted in bold letters and branching metabolites connect the boxes. Numbers represent the flux percentages of starved cultures with respect to the unstarved condition. Underlined numbers are the percentage flux of nitrogen-starved cultures, and numbers without underline are the corresponding percentages for carbon-starved cultures. Measured fluxes are distinguished from calculated fluxes by being represented in bold letters. GLC: glucose flux; SC: steady-state degradation of storage carbohydrates; EtOH: ethanol flux; DHAP: Dihydroxyacetonephosphate; GAP: Glyceraldehyde-3-phosphate, GLCi: intracellular glucose; SC: storage carbohydrates . Enzyme abbreviations are in the main text.

glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was taken as equal to the measured ethanol flux.

3.2.3 Glucose transport activity measurements

Zero-*trans* influx (the initial rate of transport before the product, intracellular glucose, builds up) of ^{14}C radiolabelled glucose was measured in a 5 second uptake assay at 30°C according to Walsh *et al.* (1994), with the modifications introduced in Chapter 2. The range of glucose concentrations was between 0.25 and 225 mM. Irreversible Michaelis-Menten equations were fitted to the results by non-linear regressing using SigmaPlot 2001 version 7.0 (SPSS Inc.).

3.2.4 Enzyme activity measurements

Enzyme extracts were prepared by sonication with glass beads at 0°C as described by van Hoek *et al.* (1998b). Enzyme activity assays were carried out on four dilutions of freshly prepared extracts through NAD(P)H-linked assays as described by van Hoek *et al.* (1998b), using a COBAS BIO (Roche, Basel) automated analyzer for spectroscopic measurements. As a control, an extraction was done in the presence and in the absence of phosphatase inhibitors (10 mM sodium fluoride and 5 mM sodium pyrophosphate), V_{max} changes were small and within the expected statistical variation.

3.2.5 Regulation Analysis

Hierarchical regulation coefficients (ρ_h) were calculated as follows:

$$\rho_h = \frac{\ln V_{max,starved} - \ln V_{max,unstarved}}{\ln J_{starved} - \ln J_{unstarved}} \tag{3.3}$$

where the subscripts *starved* and *unstarved* refer to starved (for nitrogen or carbon) or unstarved cell suspensions respectively. Each starvation experiment provided three cell suspensions, one for each condition. We performed four independent starvation experiments to measure V_{max}-values and other four to estimate fluxes through individual enzymes. The numerator of Eq. (3.3) was calculated for each starvation experiment, the values were averaged and their standard deviation computed. The average and standard deviation of the denominator was computed in the same way. Dividing average numerator and denominator yielded the average ρ_h. The metabolic regulation coefficient was calculated by subtracting ρ_h from 1.

3.3 Results

3.3.1 Steady-state fluxes

We first measured the overall steady-state fluxes of glucose, ethanol, glycerol, acetate, succinate, glycogen and trehalose, under standardized conditions of growth and starvation. Subsequently, these data were used to calculate the intracellular fluxes through the individual enzymes. *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 (referred to as "unnstarved") was washed and transferred to an anaerobic vessel with a fresh and complete medium with excess of glucose (101 mM). This condition was meant to mimic the situation of baker's yeast in dough (van Hoek *et al.*, 1998b). The above-mentioned fluxes were then measured over a period of 30 minutes. The other two batches of cells were washed and transferred to fresh medium, lacking either ammonium ("nitrogen-starved cells") or glucose ("carbon-starved cells"). After 24 hours the starved cells were harvested and the fluxes were measured in a complete medium, in the same way as was done for the unstarved cells.

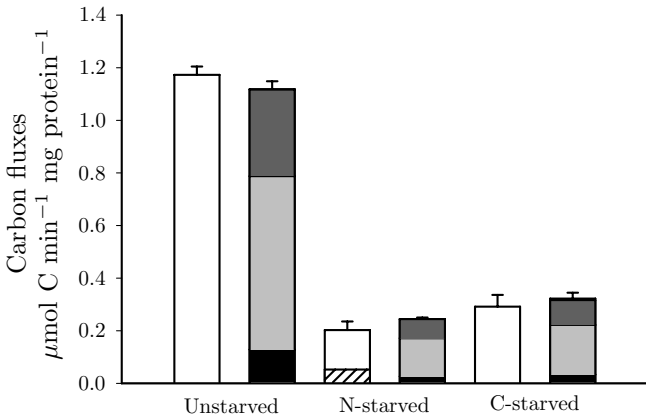

Figure 3.2: Carbon flux balances. The carbon fluxes for each condition are represented with two columns: one depicting the consumed carbon (white) and the other the produced carbon (shaded). Columns are divided into fluxes: glucose (white), storage carbohydrates (white with diagonal lines), glycerol (black), ethanol (light grey) and CO_2 (calculated from the ethanol and acetate production –dark grey). Error bars represent standard errors of the mean of the sum of consumed or produced carbon fluxes of four independent experiments carried out with different batches of cells. Glycogen was measured in only two of the experiments, their average was used as the glycogen degradation rate of the other two, in which glycogen was not measured.

Figure 3.2 and Table 3.1 show the measured fluxes. In all conditions the consumed carbon matched the produced carbon within experimental error. The production fluxes of acetate and succinate were always below 1% of the rate of glucose consumption (not shown). Nitrogen as well as carbon starvation resulted in a significant and substantial decrease of both the consumption of glucose and the production of ethanol and glycerol under the abundance conditions of the steady-state measurements assay (Student's t-test, $\alpha = 5\%$). During the starvation period, nitrogen-starved cells accumulated trehalose and glycogen. Upon transfer to complete medium, these storage carbohydrates were degraded, fueling glycolysis and contributing to the production of ethanol and glycerol.

The measured steady-state fluxes of ethanol, glycerol and glucose were used to calculate fluxes through individual enzymes in the manner detailed in the Materials and Methods section. Figure 3.1 shows the resulting fluxes of nitrogen- (underlined) and carbon-starved cultures as a percentage of those in unstarved cultures. Nutrient starvation resulted in a substantial down-regulation of the fluxes through all glycolytic and fermentative enzymes, up to more than 70 % in the case of the glucose transporters.

3.3.2 Enzyme activities

Next we asked to what extent the observed decrease of the fluxes through the glycolytic enzymes was regulated through changes of their maximum activities (V_{max}).

Table 3.1: Measured fluxes. Experimentally measured fluxes are reported in mol of the compound per minute per mg protein for each condition. Negative values represent fluxes feeding the pathway and positive values represent outgoing fluxes. Errors are standard errors of the mean for four independent experiments carried out with different batches of cells, except for glycogen. Glycogen errors are standard deviations for two independent experiments carried out with different batches of cells.

	Unstarved	**N-starved**	**C-starved**
Glucose	-0.62 ± 0.03	-0.16 ± 0.02	-0.17 ± 0.03
Glycerol	0.13 ± 0.01	0.06 ± 0.01	0.04 ± 0.00
Ethanol	1.04 ± 0.03	0.49 ± 0.05	0.33 ± 0.05
Trehalose	0.00 ± 0.00	-0.01 ± 0.00	0.00 ± 0.00
Glycogen	0.00 ± 0.00	-0.03 ± 0.01	0.00 ± 0.00

Therefore, we measured the maximum enzyme activities in unstarved cells and after 24 hours of nitrogen or carbon starvation.

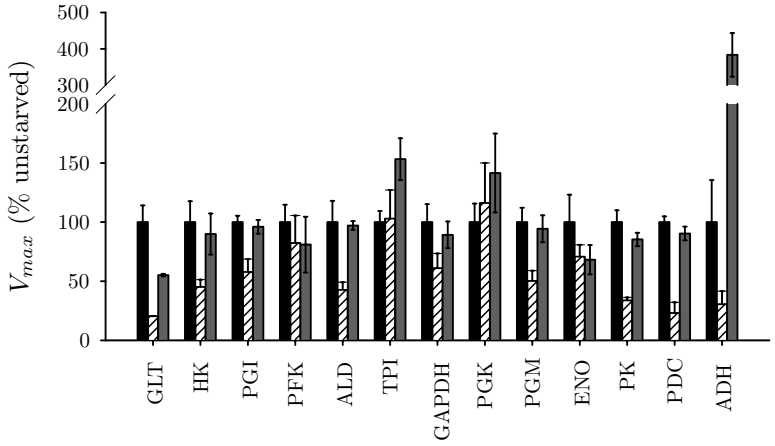

Figure 3.3: V_{max}-values as a percentage of those in unstarved cells. The percentage of V_{max}-values with respect to the unstarved condition of glycolytic and fermentative enzymes, and of the glucose transporter are shown: unstarved (black columns), nitrogen-starved (diagonally striped columns) and carbon starved (grey columns). Error bars of glycolytic and fermentative enzymes represent the percentage standard error of the mean with respect to their corresponding unstarved mean V_{max}-value, of four independent experiments carried out on different batches of cells. Error bars of the glucose transporter represent the percentage standard deviation with respect to the unstarved mean V_{max}-value of two independent experiments carried out on different batches of cells.

Nutrient starvation resulted in changes of V_{max} that varied in extent and direction (Figure 3.3). The cells responded in a very different way to the two types of starvation. During nitrogen starvation, the activities of GLT, HK, PGI, ALD, phosphoglycerate mutase (PGM), pyruvate kinase (PK), pyruvate decarboxylase (PDC) and alcohol dehydrogenase (ADH) were down-regulated (Student's t-test $\alpha = 5\%$), while the

other enzyme activities remained unchanged within statistical error. During carbon starvation, on the other hand, only the V_{max} of the glucose transporter decreased significantly, while the maximum activities of triosephosphate isomerase (TPI) and ADH increased (Student's t-test $\alpha = 5\%$).

3.3.3 Regulation Analysis

Table 3.2: Hierarchical and metabolic regulation coefficients of nitrogen and carbon starvations. Hierarchical and metabolic regulation coefficients were calculated as described in the Materials and Methods section. Errors are standard errors of the mean (SEM) calculated for four independent V_{max} measurements and four independent flux estimations, all performed upon independent batches of cells.

	Nitrogen starvation			Carbon starvation		
Enzyme	ρ_h	SEM	ρ_m	ρ_m	SEM	ρ_m
GLT	1.2	0.1	-0.2	0.4	0.1	0.6
HK	1.0	0.2	0.0	0.1	0.0	0.9
PGI	0.8	0.3	0.2	0.0	0.0	1.0
PFK	0.4	0.2	0.6	0.4	0.4	0.6
ALD	1.1	0.5	-0.1	0.0	0.2	1.0
TPI	0.1	0.9	0.9	-0.4	0.2	1.4
GAPDH	0.7	0.5	0.3	0.1	0.0	0.9
PGK	0.0	0.2	1.0	-0.3	0.1	1.3
PGM	1.0	0.4	0.0	0.0	0.0	1.0
ENO	0.4	0.5	0.6	0.3	0.1	0.7
PK	1.4	0.3	-0.4	0.1	0.0	0.9
PDC	2.3	0.6	-1.3	0.1	0.0	0.9
ADH	1.7	0.4	-0.7	-1.3	0.2	2.3

Nutrient starvation resulted in decreased fluxes and a variety of V_{max} changes. In order to dissect the extent to which the changes of V_{max} were responsible for the flux changes from the extent to which the fluxes were rather regulated by changes in their interaction with the rest of metabolism, we calculated the hierarchical and metabolic regulation coefficients (*cf.* Eqs. (3.2) and (3.3). The results are shown in Table 3.2. The hierarchical regulation coefficients, ρ_h, ranged between -1.3 and 2.2, spanning all categories of regulation (*cf* Chapter 2). This wide variation of iρ_h is not a matter of statistical variation (*cf.* Table 3.2).

We distinguish the following categories of regulation:

- **Purely hierarchical regulation:** During nitrogen starvation the hierarchical regulation coefficient (ρ_h) of a number of enzymes was not significantly different from 1. Since the metabolic and the hierarchical regulation coefficients sum up to 1 [Eq. (3.1)], these enzymes had a metabolic regulation coefficient (ρ_h) not significantly different from 0. This implied that the change of flux was regulated predominantly by the change in V_{max}, while the interaction with the rest of metabolism made a negligible contribution. HK and PGM were the clearest examples of this type of regulation.

- **Purely metabolic regulation:** Enzymes with ρ_h not significantly different from zero were found in both types of starvation. For these enzymes, the flux was predominantly regulated by the interaction with the rest of metabolism without any significant contribution of changes in V_{max}. PGK in nitrogen starvation, and PGI, ALD and PGM in carbon starvation were the clearest examples of this category.
- **Cooperative regulation:** a number of enzymes were regulated cooperatively by changes in V_{max} and changes in their interaction with the rest of metabolism. This was reflected by a ρ_h value between 0 and 1 and significantly different from both 0 and 1. PFK was regulated in this way during nitrogen starvation, and so were GLT and ENO during carbon starvation.
- **Antagonistic regulation, directed by metabolism:** Negative ρ_h values result when the flux changes in opposite direction to the V_{max}. In these cases ρ_m was larger than 1, implying that the metabolic regulation dominated and was counteracted by hierarchical regulation, which acted "conservatively" in that it attempted to keep to antagonize the flux change. The regulation of ADH during carbon starvation was an outstanding instance of this category.
- **Antagonistic regulation, directed by V_{max}:** This category is the opposite of the previous. ρ_h exceeded 1 and ρ_m was therefore negative. In these cases, the changes in the interaction with the rest of metabolism and the changes of V_{max} again counteracted each other, but now the change of V_{max} dominated the outcome, with the metabolic regulation acting conservatively. Only nitrogen starvation showed enzyme fluxes regulated in this way. PDC and ADH were the most conspicuous cases. Also GLT was classified in this category, but it should be noticed that its ρ_h was very close to 1, meaning that its regulation was predominantly hierarchical with a small, but significant, antagonistic contribution of the interaction with the rest of metabolism.

3.4 Discussion

Nutrient starvation of the yeast *S. cerevisiae* resulted in decreased glucose consumption and decreased ethanol and glycerol production (Nilsson *et al.*, 2001b; Rossell *et al.*, 2002; Thomsson *et al.*, 2003, this study). These flux decreases were accompanied by a limited variety of changes in the maximum activities of glycolytic and fermentative enzymes. The changes differed in magnitude and direction among enzymes, and the profile of these changes differed between starvation types (Nilsson *et al.*, 2001b; Thomsson *et al.*, 2003, this study). Similar findings have been reported for other transitions, such as changes of dilutions rates in chemostat cultures (van Hoek *et al.*, 1998b) or shifts between different growth limitations (Daran-Lapujade *et al.*, 2004).

Our goal was to understand the regulation of metabolic fluxes by the concerted action of gene expression and metabolic interactions. There are two related, but different aspects to this problem. On the one hand, there is the local regulation of fluxes through individual enzymes, and on the other hand, there is the extent to which

this local regulation influences the pathway's (global) flux. In this contribution we expound how the first aspect can be understood. Using Regulation Analysis, we determined experimentally the type of regulation of fluxes through individual glycolytic and fermentative enzymes, as yeast was responding to nutrient starvation. Others and we have applied this method previously to the regulation of flux through some steps in a pathway (ter Kuile & Westerhoff, 2001; Even *et al.*, 2003, Chapter 2). The present paper reports the first comprehensive study extending Regulation Analysis to all enzymes in a complete metabolic pathway. It is therefore the first time that we could address the validity of a number of existing paradigms of metabolic regulation of pathway flux.

In the Introduction we distinguished three regulatory paradigms, *i.e.* single enzyme, multi-site, or all metabolic. If we translate these paradigms to the terminology of Regulation Analysis, single-enzyme regulation implies that one enzyme is regulated in a purely hierarchical manner ($\rho_h = 1$), while all the others are regulated only through metabolism ($\rho_h = 0$). Neither our results nor that of others are compatible with single-enzyme regulation (Nilsson *et al.*, 2001b; Thomsson *et al.*, 2003; van Hoek *et al.*, 1998b; Daran-Lapujade *et al.*, 2004).

The hypothesis of multi-site modulation proposes metabolite homeostasis as a constraint to metabolic flux regulation, excluding the possibility of metabolic regulation. This corresponds to a situation where $\rho_h = 1$ for all enzymes. In our carbon starvation experiments, however, a number of fluxes through individual enzymes was regulated exclusively by the interaction of the enzyme with the rest of metabolism ($\rho_h = 0$). Among these enzymes, PGI and ALD have a unique isoenzyme form, excluding that this apparent metabolic regulation is actually caused by K_m changes through the expression of isoenzymes. During nitrogen starvation, unspecific degradation of proteins via autophagy is enhanced (Abeliovich & Klionsky, 2001) and therefore one might have expected a proportional decrease of all enzyme amounts (corresponding to multi-site modulation). We observed, however, disproportional changes in enzyme activities (ρ_h's unequal to each other and $\neq 1$). Protein degradation is therefore unlikely to be the sole cause of these enzyme activity changes.

The third paradigm, exclusively metabolic regulation, would correspond to all $\rho_h = 0$. Our results are incompatible with this hypothesis.

If none of these three regulatory paradigms holds true, how should we then envisage regulation? Within a single pathway, fluxes through individual enzymes were regulated in different ways, suggesting that enzymes play different roles in the regulation of the pathway's flux. Changes in fluxes through some enzymes were caused predominantly by changes in enzyme activities (ρ_h close to or above 1). The interaction with the rest of metabolism either complied ($\rho_m = 0$) or antagonized, diminishing the effect of the enzyme activity change on the local flux ($\rho_m < 0$). In these cases, enzyme activity changes seemed to "lead" the regulatory response, while the compliance or antagonism of the interaction with the rest of metabolism constituted the system's response to this "lead". Other enzyme fluxes were regulated with small or no change in enzyme activities ($0 < \rho_h < 1$ and $\rho_h = 0$, respectively). These enzymes seemed to "follow" the "leader" enzymes by adjusting their rate through their interaction with the rest of metabolism. Yet other enzymes changed their maximum activity in opposite direction to the change in flux ($\rho_h < 0$). These "conservative" enzymes seemed

to "pull back", to restrain the regulation by the leading enzymes. Interestingly, the pathway's regulation profile differed radically between the two types of starvation. The sets of enzymes leading the regulatory response, as well as those following or pulling back, differed between starvation types. Apparently, the regulatory roles of enzymes are not fixed properties, but rather change when cells are challenged in different ways. And this finding is what we should like to propose as a new paradigm for metabolic regulation, regulation is diverse within a pathway, some enzymes taking the lead, others helping and yet others acting conservatively.

Several experimental limitations limit further conclusions from and indeed the accuracy of our analysis. For one, some of the calculated regulation coefficients presented in Table 3.2 have relatively high standard errors of the mean. This is due to the necessary (because we are studying regulation) consideration of *changes* of fluxes and enzyme activities instead of absolute values in their calculation. The large errors limit the application of Regulation Analysis, at this moment, to the analysis of perturbations that cause relatively large changes of flux and stresses upon the necessity to further develop the reproducibility of analytical techniques as well as of cultivation and sampling procedures. However, the large errors do not impede an unambiguous classification of most enzymes in different regulation categories, proving that these classifications are not just theoretical possibilities, but actual ways by which living cells regulate fluxes through individual enzymes. And here Regulation Analysis differs from Metabolic Control Analysis in that Regulation Analysis does not require changes to be small.

Another crucial issue is a correct estimation of the local fluxes. Based on measured fluxes (Table 3.1) we calculated the fluxes through individual glycolytic and fermentative enzymes using the simplified scheme depicted in Figure 3.1. We neglected the branching fluxes through the pentose phosphate pathway as well as through the anabolic pathways. Although the differences between produced and consumed carbon are not statistically significant (Figure 3.2), mean consumed carbon was in excess with respect to produced carbon in unstarved cultures, while the reverse was suggested for starved cultures. Since absolute growth during our 30 minutes assay is undetectable, we estimated the fraction of glycolytic flux diverged into biomass. As the biomass yield for optimal anaerobic growth is 0.1 g biomass per g glucose and the carbon content of biomass is 40 % (Verduyn *et al.*, 1991), at most 10 % of the glucose may be incorporated into biomass. Because *S. cerevisiae* lacks a transhydrogenase, the pools of NADPH and NADH are not linked (Bakker *et al.*, 2001). For each mole of glucose going into biomass, one mole of NADPH is required (Verduyn *et al.*, 1990b). If 10% of the glucose is utilized for biomass production, then at most 5% of the glucose flows through the phosphogluconate pathway (2 NADPH are produced per glucose 6-phosphate rerouted). Based on these calculations we neglected the branches into the pentose phosphate pathway and into anabolism: Taking them into account would not change our regulation coefficients such that our above conclusions would change.

Another simplification in Figure 3.1 was the exclusion of fructose-1,6-bisphosphatase. This enzyme may cause substantial futile cycling, particularly in the transition from carbon starved media to complete media (Shulman & den Hollander, 2004). However, in our experimental conditions the activity of fructose-1,6-bisphosphatase was very low in all cultures (below 0.002 μmol min^{-1} mg protein^{-1}). In contrast to

Shulman & den Hollander (2004), our starved cells were not adapted to growth on acetate or another gluconeogenic substrate: The required stimuli for triggering the expression of fructose-1,6-bisphosphatase may have been absent under our experimental conditions.

It is possible to extend Regulation Analysis to dissect the different processes within the hierarchical component further (Westerhoff *et al.*, 2000). Such a comprehensive study is underway. In a pilot experiment, we investigated whether any change in V_{max} occurred at 15 minutes after the transfer of starved cells into complete medium, the timescale at which covalent modifications may occur. The changes in V_{max} we measured were not beyond what was to be expected on the bases of statistical variation, with the exception of PK. We observed an activation of PK in nitrogen and carbon starved cultures, 6 and 16 fold, respectively (result not shown). Indeed PK has been reported to be activated through phosphorylation by protein kinase A (Portela *et al.*, 2002).

Concerning the regulation of the pathway's flux, our results suggest that changes outside glycolysis contributed to the decrease of glycolytic flux in carbon starved cells, regulating glycolytic enzymes metabolically. The only V_{max} that decreased significantly was that of the glucose transporter. However, the decrease of the transporter V_{max} was only 40% of the decrease of the flux. The remaining 60% of metabolic regulation is unlikely to be initiated by any of the other enzymes in the pathway, since no other V_{max} decreased significantly. Thus, part of the metabolic regulation of glycolytic and fermentative enzymes during carbon starvation must have originated outside the pathway. We measured the concentrations of two obvious candidates, ATP and ADP (not shown). Their ratio did not change (0.7), but changes in the total concentration of the summed adenine nucleotides (ATP + ADP + AMP) may still be involved in the decrease of glycolytic flux.

Our results and analysis have shown that pathway fluxes may be regulated not only through expression of enzymes within the pathway, but also through metabolic regulation that may be elicited by changes foreign to the pathway in study. Thus, our findings highlight the need to integrate transcriptome and proteome analyses with other levels of regulation, including the metabolic, and to do this quantitatively. Using Regulation Analysis we have described the regulation of steady-state fluxes through individual enzymes unraveling a previously undescribed complexity of flux regulation. The diversity of regulation within a common pathway suggests that enzymes play a limited number of different regulatory roles. We suggest an alternative mechanism for flux modulation. A mechanism in which regulation is not exclusively hierarchical as in multi-site modulation nor effected by a single regulatory enzyme, but involves different regulatory roles for each enzyme, and a plasticity that allows these roles to shift between enzymes when the cell is confronted with different challenges. Our findings invite to reconsider our views on regulatory processes. Regulation of metabolic fluxes needs not to be governed by single drives or constraints, but may result from a combination of them and their relative importance may well vary between challenges.

Acnowledgements

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Chapter 4

The Regulation of Enzyme Rates and Catalytic Capacities

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 and 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. The more than 10-fold increase in the fluxes through the glycolytic enzymes was for 50 – 80% due to metabolic regulation, the percentages varying between the various steps of glycolysis and between the cultivation conditions tested. Within the 50 – 20% hierarchical regulation of fluxes, transcription played a minor role while regulation of protein synthesis or degradation was predicted to be the most important. These were also predicted to account for 75% - 100% to the regulation of protein levels.

4.1 Introduction

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 as well as accurate estimates of metabolic flux distributions (deRisi *et al.*, 1997; Washburn *et al.*, 2001; Forster *et al.*, 2003). The

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central dogma of molecular biology is that DNA encodes mRNA and that mRNA encodes proteins, which in turn fulfill the many functions in the cell. Therefore, a strong correlation was anticipated between 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 (Griffin *et al.*, 2002; Ideker *et al.*, 2001; Greenbaum *et al.*, 2003; Bro *et al.*, 2003), between mRNA and *in vivo* fluxes (Even *et al.*, 2003; Daran-Lapujade *et al.*, 2004; Yang *et al.*, 2002) and between enzyme activities and fluxes (ter Kuile & Westerhoff, 2001, Chapter 3) 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 (Piper *et al.*, 2002; Bro *et al.*, 2003). 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 are growing in a constant environment for prolonged periods of time, mRNA levels, protein concentrations/activities and fluxes correlated poorly (ter Kuile & Westerhoff, 2001; Daran-Lapujade *et al.*, 2004; Kolkman *et al.*, 2006). 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 turn-over in *Saccharomyces cerevisiae*, showed that these varied significantly between proteins and conditions (Arava *et al.*, 2003; Pratt *et al.*, 2002; Smirnova *et al.*, 2005). Posttranslational modifications of proteins and metabolic regulation need to be considered as well (Chapters 2 and 3).

The question then becomes how one should quantify and integrate all these different levels of regulation so as to come to a coherent understanding of the regulation of cell function. This question is addressed by Regulation Analysis (ter Kuile & Westerhoff, 2001, Chapters 2 and 3). 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:

$$\frac{\Delta \ln f(e)}{\Delta \ln J} + \frac{\Delta \ln g(\mathbf{x}, \mathbf{k})}{\Delta \ln J} = \rho_h + \rho_m = 1 \tag{4.1}$$

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. (4.1), ρ_h is the "hierarchical regulation coefficient" that quantifies to which 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, all the way 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 as well as through changes in the affinity of the isoenzymes, *e.g.* through shifts in isoenzyme expression (Chapter 2). In practice, the hierarchical regulation coefficient ρ_h is readily determined whenever the V_{max} as well as the flux J through the enzyme can be measured or estimated. The metabolic regulation coefficient ρ_m then follows from the summation theorem expressed in Eq. (4.1).

As presented, this method does not enable the quantification of the contribution of transcription, mRNA degradation, 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 extended Regulation Analysis is applied to glycolysis in the yeast *Saccharomyces cerevisiae.* Although yeast glycolysis is one of the most extensively studied metabolic pathways, it is largely unresolved how yeast regulates the V_{max} of its enzymes. Regulation of the expression of the glycolytic enzymes will be investigated in two important situations that result in drastic changes in the glycolytic fluxes: (i) the shift from respiratory to fermentative metabolism resulting from the presence or absence of oxygen, and (ii) the energetic workload imposed by an uncoupling agent, *i.e.* benzoic acid. To quantify the regulation of the V_{max}'s and enzyme rates by the different levels of gene expression, we measured the changes of glycolytic enzyme rates, V_{max} values, protein concentrations and mRNA concentrations when yeast was exposed to these challenges.

4.2 Materials and Methods

4.2.1 Strain and growth conditions

The haploid, prototrophic *Saccharomyces cerevisiae* strain CEN.PK113-7D (*MAT*a, *MAL2-8*^{c}, *SUC2*, obtained from P. Kötter, Frankfurt, Germany) was grown at 30°C in 2-liter fermenters (Applikon) as described in (Verduyn *et al.*, 1992). Briefly the chemostats were fed with a defined mineral medium (Verduyn *et al.*, 1992) in which glucose was the growth-limiting nutrient with all other nutrients in excess. The dilution rate (equal to the specific growth rate in steady-state cultures) was set at 0.10 h^{-1}. The pH was kept constant at 5.0. Aerobic cultures were flushed with air while anaerobic cultures were flushed with pure nitrogen gas (5.0; Hoekloos). For anaerobic cultivations, the medium was supplemented with Tween-80 and ergosterol (0.42 g l^{-1} and 10 mg l^{-1} respectively), which are essential for anaerobic growth (Andreassen & Stier, 1953). For the anaerobic chemostats with benzoic acid, 2 mM sodium benzoate were added to the feed medium. Chemostat cultures were assumed to be in steady state when, after at least five volume changes, the culture dry weight, specific carbon-dioxide production rate, oxygen-consumption rate (for aerobic cultures) and production rate of extracellular metabolites (for anaerobic cultures) changed by less than 2% during 24 hours. Steady-state samples were taken after 10 to 14 volume changes to avoid strain adaptation due to long-term cultivation (Jansen *et al.*, 2005).

Table 4.1: Main physiological characterisitics of the cultures used in this study. Entries represent the average and standard error of the mean of the indicated amount of independent chemostat steady states. Y_{SX} the yield of biomass (g dry biomass per g glucose consumed). q indicate biomass specific rates with the units $mmol(g\,dry\,biomass)^{-1}\,h^{-1}$. The carbon recoveries were calculted using the yields of biomass, ethanol, carbon dioxide and glycerol. BDL stands for "below detection limit".

	Aerobic	**Anaerobic**	**Benzoate**
Glucose in feed (mM)	41.4 ± 0.1	138 ± 2	143 ± 1
Residual Glucose (mM)	BDL	0.26 ± 0.05	1.8 ± 0.2
Y_{SX} ($g\,g^{-1}$)	0.49 ± 0.02	0.094 ± 0.001	0.045 ± 0.001
$q_{glucose}$	1.15 ± 0.0	6.2 ± 0.1	12.3 ± 0.2
$q_{ethanol}$	BDL	9.6 ± 0.2	21.5 ± 0.6
$q_{glycerol}$	BDL	0.8 ± 0.0	0.9 ± 0.1
q_{O_2}	2.47 ± 0.00	BDL	BDL
q_{CO_2}	2.85 ± 0.1	10.3 ± 0.3	22.9 ± 0.3
Carbon recovery (%)	101 ± 1	99 ± 0	100 ± 1

4.2.2 Analytical methods

Culture supernatants and media were analyzed by HPLC and culture dry weights were determined as described by Postma *et al.* (1989).

4.2.3 Microarray analysis

Sampling of cells from chemostats, probe preparation and hybridization to Affymetrix GeneChip microarrays were performed as previously described (Piper *et al.*, 2002). The results were derived from five independent aerobic cultures, four independent anaerobic cultures and three independent anaerobic cultures with benzoic acid. Acquisition and quantification of array images was performed using the Affymetrix software package Microarray Suite v5.0.

4.2.4 Proteins analysis by LC/MS-MS

The detailed protocol and statistical analysis can be found in de Groot et al. (submitted to *Microbiology*). Briefly aerobic and anaerobic chemostats were grown both with ^{14}N- and ^{15}N-labelled ammonium sulphate as the sole nitrogen source as described above. Proteins from ^{14}N- and ^{15}N-metabolically labelled yeasts were extracted and mixed. An SDS-page gel was ran to separate the proteins and cut in 40 slices. After in-gel protein digestion using trypsin, the resulting tryptic digests were analyzed in triplicate by nanoflow-LC-MS/MS. Proteins were identified using MS/MS. Subsequently the relative protein expression levels were obtained by comparing the extracted ion chromatograms of the co-eluting ^{14}N and ^{15}N labelled peptide pairs. Proteins were quantified based on two independent culture replicates and three analytical replicates.

Table 4.2: Regulation of glycolytic fluxes and V_{max}'s in yeast in anaerobic compared to aerobic glucose-limited chemostats. Hierarchical (ρ_h) and metabolic (ρ_m) regulation coefficients were calculated as described in the Materials and Methods section. The regulation of V_{max} by mRNA changes ($\rho_{mRNA,V_{max}}$) where calculated as indicated by the corresponding term in Eq. (4.11). The regulation of fluxes through individual enzymes by mRNA changes ($\rho_{mRNA,flux}$) was calculated accoridnt to Eq. (4.12). The regulation of V_{max} by posttranslational modifications was calculated according to its corresponding term in Eq. (4.11). Errors are standard errors of the mean (SEM) calculated for the corresponding number of independent measurements indicated in the Materials and Methdods section.

	ρ_h	SEM	ρ_m	$\rho_{mRNA,Vmax}$	SEM	$\rho_{mRNA,flux}$	SEM	$\rho_{PT,Vmax}$	SEM
HK	0.5	0.06	0.5	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		
ALD	-0.24	0.05	1.24	0.25	0.28	-0.06	0.07	2.2	0.73
TPI	-0.72	0.07	1.72	0.16	0.13	-0.11	0.1	1.13	0.63
GAPDH	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
PK	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				

Table 4.3: Regulation of glycolytic V_{max}'s and fluxes in anaerobic, glucose-limited chemostats with versus without benzoic acid. Hierarchical (ρ_h) and metabolic (ρ_m) regulation coefficients were calculated as described in the Materials and Methods section. The regualtion of V_{max} by mRNA changes ($\rho_{mRNA,V_{max}}$) were calculated as indicated by the corresponding term in Eq. (4.11). The regulation of fluxes through individual enzymes by mRNA changes ($\rho_{mRNA,flux}$) was calculated according to Eq. (4.12). Errors are standard errors of the mean (SEM) calculated for the corresponding number of independent measurements indicated in the Materials and Methdods section.

	ρ_h	SEM	ρ_m	$\rho_{mRNA,Vmax}$	SEM	$\rho_{mRNA,flux}$	SEM
HK	-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
ALD	0.93	0.15	0.07	0.35	0.2	0.32	0.18
TPI	2.12	0.16	-1.12	0.12	0.08	0.26	0.18
GAPDH	-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	0.2
PGM	0.17	0.1	0.83	1.07	0.78	0.18	0.08
ENO	0.27	0.08	0.73	0.73	0.34	0.2	0.07
PK	0.95	0.11	0.05	0.27	0.1	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	0.1	0.06

4.2.5 Enzyme assays

Glycolytic enzyme activities and protein concentrations in freshly prepared cell extracts were assayed according to Jansen *et al.* (2005), with the exception of triosephosphate isomerase for which the assay mixture contained Triethanolamine-HCl buffer (pH 8.2), 200 mM; NADH, 0.15 mM; glycerol-3P-dehydrogenase (Roche), 8.5 U.ml^{-1}; glyceraldehyde-3-phosphate, 17.4 mM. The enzyme activities presented in this work are the average of measurements in samples from at least three independent culture replicates.

4.2.6 Metabolic flux distribution

Intracellular metabolic fluxes in growing cells were calculated through metabolic flux balancing using the compartmented stoichiometric model described in (Daran-Lapujade *et al.*, 2004).

4.2.7 Regulation Analysis

For any reaction step in glycolysis, hierarchical regulation coefficients ρ_h (ter Kuile & Westerhoff, 2001, Chapter 2) were calculated as follows:

$$\rho_h = \frac{\ln\langle V_{max,i}\rangle - \ln\langle V_{max,j}\rangle}{\ln\langle J_i\rangle - \ln\langle J_j\rangle} \tag{4.2}$$

where angel brackets enclose mean values, the subscripts i and j refer to different growth conditions and J refers to steady-state fluxes. The mean fluxes through individual glycolytic enzymes and their standard deviations ($n \geqslant 4$) and the mean V_{max}-values and the standard deviations of all individual glycolytic enzymes were computed ($n \geqslant 3$). The mean values were translated into logarithmic space and the hierarchical regulation coefficient was computed as shown in Eq. (4.2).

Calculation of the standard deviation and standard error of the mean of hierarchical regulation coefficients

The calculation of intracellular fluxes using a compartmentalized model uses mean measured extracellular concentrations and flows with their standard deviations. Because the calculated intracellular fluxes are reported as mean values with their standard deviations, the calculation of the standard deviations of the hierarchical regulation coefficient requires additional computations. The standard deviation of the numerator of Eq. (4.2) was calculated as follows:

$$SD_{num} = \sqrt{\left[\frac{\ln\langle V_{max,i}\rangle}{\ln(\langle V_{max,i}\rangle - SD_{V_{max,i}})}\right]^2 + \left[\frac{\ln\langle V_{max,j}\rangle}{\ln(\langle V_{max,j}\rangle - SD_{V_{max,j}})}\right]^2} \tag{4.3}$$

where the subscript *num* refers to the numerator of Eq. (4.2), $SD_{V_{max,i}}$ and $SD_{V_{max,j}}$ are the standard deviations, in Cartesian space, of the V_{max} values of conditions i and

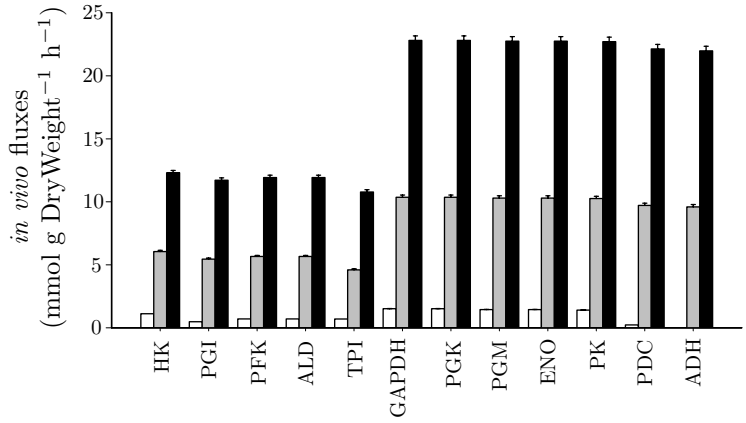

Figure 4.1: *in vivo* fluxes of *S. cerevisiae*'s glycolysis cultivated in glucose-limited chemostats in aerobiosis or anaerobiosis in the presence or absence of benzoic acid calculated from measured external metabolites using the stoichiometric model described in (Daran-Lapujade *et al.*, 2004). White bars represent aerobic cultures, grey bars anaerobic cultures and black bars anaerobic cultures with benzoate.

j, respectively. Similarly the standard deviation of the denominator was calculated with:

$$SD_{deno} = \sqrt{\left[\frac{\ln\langle J_i\rangle}{\ln(\langle Ji\rangle - SD_{J_i})}\right]^2 + \left[\frac{\ln\langle J_j\rangle}{\ln(\langle J_j\rangle - SD_{J_j})}\right]^2} \tag{4.4}$$

where the subscript *deno* refers to the denominator of Eq. (4.2), SD_{J_i} and SD_{J_j} are the standard deviations, in Cartesian space, of the flux values of conditions i, and j, respectively. Since symmetric error bars become asymmetric in logarithmic space and the lower bound error is larger than the upper one, this procedure leads to an overestimation of the standard deviation in the positive direction. The standard deviation of the hierarchical regulation coefficient can be computed in the following way:

$$SD_{\rho_h} = \rho_h \cdot \sqrt{\left(\frac{SD_{num}}{num}\right)^2 + \left(\frac{SD_{deno}}{deno}\right)^2} \tag{4.5}$$

where *num* and *deno* represent the values of the numerator and denominator of Eq. (4.1), respectively. The standard error of the mean (SEM, Tables 4.2 and 4.3) were calculated by dividing the standard deviation by the square root of 3.

4.3 Results

4.3.1 Hierarchical and metabolic regulation of glycolytic enzyme rates

In all steady-state glucose-limited chemostat experiments, *S. cerevisiae* was cultivated at the same dilution rate, and thus specific growth rate, of 0.10 h^{-1}. The

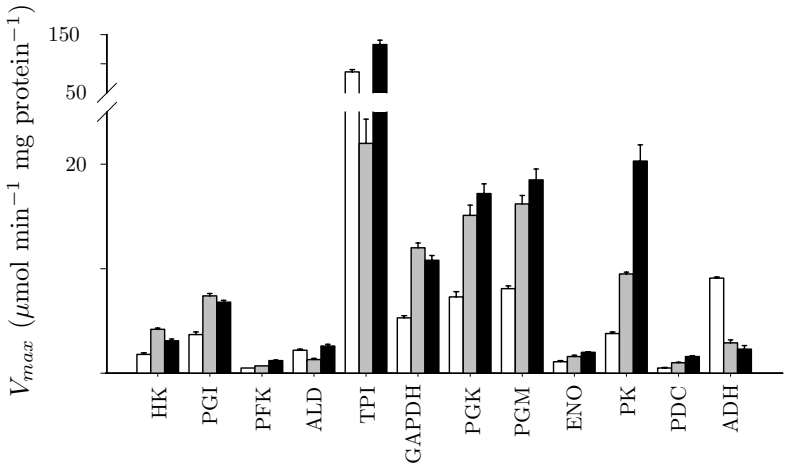

Figure 4.2: *in vitro* determined enzyme capicities (V_{max}) of the glycolytic enzymes of *S. cerevisiae* cultivated in glucose-limited chemostats in aerobiosis or anaerobiosis with or without benzoic acid. White bars represent aerobic cultures, grey bars anaerobic cultures and black bars anaerobic cultures with benzoate.

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 (Table 4.1), while 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 (Table 4.1).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 (Figure 4.1). To further increase the carbon fluxes in glycolysis, the non-metabolizable weak acid benzoate was added. Weak acids cause an increased ATP requirement that is met by increased rates of glucose dissimilation (Verduyn *et al.*, 1992). This was reflected in a two-fold increase in the fluxes through the glycolytic enzymes when 2 mM benzoic acid was added to anaerobic cultures (Figure 4.1).

In order 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 (so-called hierarchical regulation coefficient ρ_h) was determined using Regulation Analysis (Tables 4.2 and 4.3, column 1). A reaction that is purely regulated by the cascade of gene expression would have a ρ_h of 1. Conversely a reaction

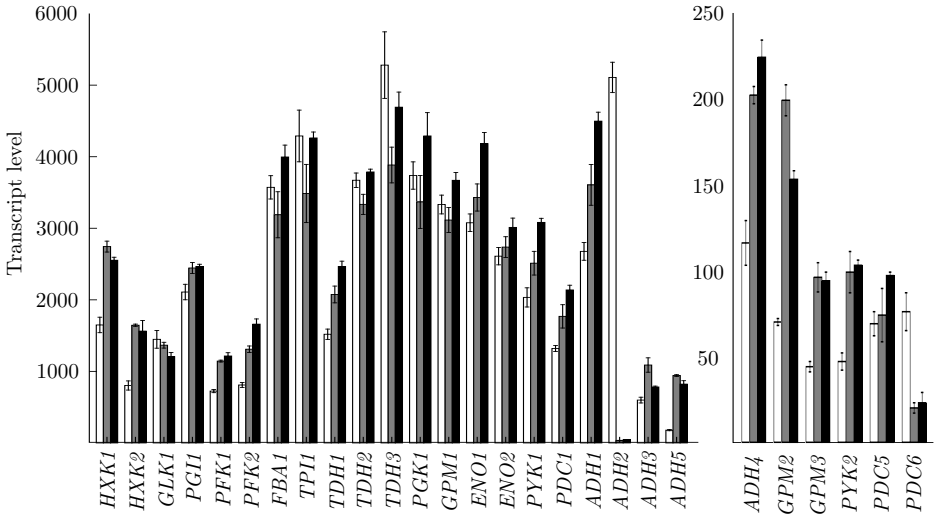

Figure 4.3: Transcript levels of the mRNAs of glycolytic enzymes of *S. cerevisiae* cultivated in glucose-limited chemostats in aerobiosis or anaerobiosis with or without benzoic acid. White bars represent aerobic cultures, grey bars anaerobic cultures and black bars anaerobic cultures with benzoate.

that is solely metabolically regulated would have a ρ_h of 0, see Eq. (4.1). In most cases, the changes in fluxes resulted from both hierarchical and metabolic regulatory mechanisms, but their respective contribution was clearly depending on the reaction considered and on the culture conditions (Table 4.2 and 4.3, columns 1 and 3). 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% of the enzyme rate regulation (Table 4.2, column 1). The remaining 50 – 80% of the flux changes was caused by metabolic regulation (Table 4.2, 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 while most V_{max} values hardly changed (Figure 4.1 and 4.2). 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 4.3). There were a few notable exceptions. The V_{max} of PFK, ALD, TPI and PK increased strongly resulting in large hierarchical regulation coefficients for these enzymes.

4.3.2 Dissecting hierarchical regulation into contributions by transcription, translation, protein degradation and post-translational 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 analyse the

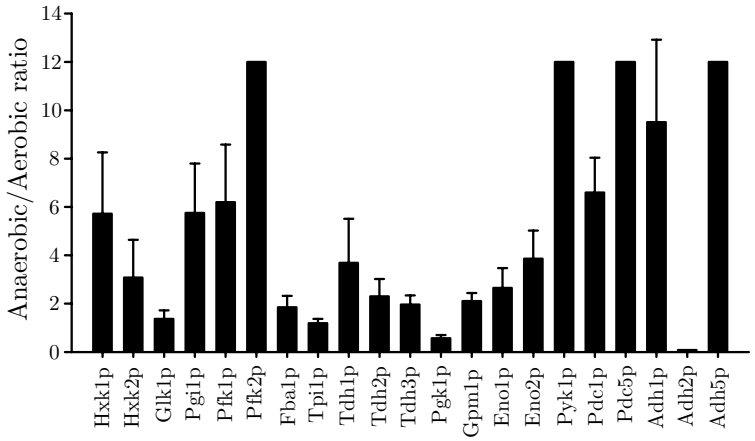

Figure 4.4: Ratio of the protein concentrations measured in aerobic and anaerobic cultures without benzoic acid of glycolytic proteins from *S. cerevisiae*.

hierarchical regulation of V_{max} in more detail Regulation Analysis was extended as follows.

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

$$V_{max} = k_{cat}[protein] \tag{4.6}$$

Since the cultures under study were at steady state, the rate of translation v_{trans} should equal the rate of protein degradation plus the dilution of proteins due to cell growth *i.e.*:

$$\frac{\mathrm{d}[protein]}{\mathrm{d}t} = v_{trans} - v_{deg} - v_{dil} = 0 \tag{4.7}$$

where v_{trans} represents the rate of translation, v_{deg} the rate of protein degradation and v_{dil} the rate of protein dilution due to growth.

The rate of synthesis of any specific protein v_{trans} was approximated by:

$$v_{trans} = k_{trans} \cdot [ribosome] \cdot [mRNA] \tag{4.8}$$

where k_{trans} a first order kinetic constant of the rate of translation, which 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 were taken proportional to the concentration of that protein with proportionality constants μ (the specific growth rate) and k_{deg}, respectively, *i.e.*:

$$\frac{\mathrm{d}[protein]}{\mathrm{d}t} = k_{trans} \cdot [ribosome] \cdot [mRNA] - k_{deg} \cdot [protein] - \mu \cdot [protein] = 0 \tag{4.9}$$

Combining Eq. (4.9) with Eq. (4.6) yields:

$$V_{max} = \frac{k_{cat}\, k_{trans}\, [ribosome]\, [mRNA]}{\mu + k_{deg}} \tag{4.10}$$

The contribution of each of the components in Eq. (4.10) to an overall change of V_{max} of the enzyme was determined by taking its logarithm, calculating the difference between two conditions and dividing by $\Delta \ln V_{max}$:

$$\begin{aligned} 1 = \underbrace{\frac{\Delta \ln k_{cat}}{\Delta \ln V_{max}}}_{PT\ modification} &+ \underbrace{\left(\frac{\Delta \ln k_{trans}}{\Delta \ln V_{max}} + \frac{\Delta \ln [ribosome]}{\Delta \ln V_{max}} \right)}_{translation} \\ &+ \underbrace{\frac{\Delta \ln [mRNA]}{\Delta \ln V_{max}}}_{transcription} - \underbrace{\frac{\Delta \ln (\mu + k_{deg})}{\Delta \ln V_{max}}}_{degradation/dilution} \\ &\equiv \rho_{PT,V_{max}} + \rho_{mRNA,V_{max}} + \rho_{trans,V_{max}} + \rho_{dd,V_{max}} \end{aligned} \tag{4.11}$$

in which $\rho_{PT,V_{max}}$ quantifies the regulation of V_{max} by posttranslational modifications, $\rho_{trans,V_{max}}$ the regulation of V_{max} by translation activity, $\rho_{mRNA,V_{max}}$ quantifies the regulation of V_{max} by the mRNA concentration, and $\rho_{dd,V_{max}}$ 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 $\rho_{trans,V_{max}}$. 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. Since the specific growth rate is measured easily, it can be incorporated explicitly in the determination of $\rho_{dd,V_{max}}$.

4.3.3 Regulation of enzyme rates and V_{max}'s by mRNA concentration changes

In order to quantify the importance of transcriptional regulation within the gene expression cascade ($\rho_{mRNA,V_{max}}$) the mRNA levels of the 27 genes encoding the isoenzymes of glycolysis and fermentation were measured by microarray analysis (Figure 4.3). The adaptation to anaerobiosis in the presence or absence of benzoic acid did not result in significant changes in the expression of 9 of the 27 genes glcolytic genes (*GLK1, PGI1, FBA1, TPI1, THD2, THD3, PGK2, GPM1* and *ENO2*). *ADH2* and *PDC6* were significantly down-regulated in the presence and in the absence of benzoic acid. *HXK2* was upregulated only in the absence of benzoic acid, while *TDH1, ENO1, PYK1, PDC1, ADH1* and *PDC5* were upregulated only in its presence. The remaining genes (*HXK1, PFK1, PFK2, ADH3, ADH5, GPM2, GPM3, PYK2* and *ADH4*) were up-regulated both in the presence and in the absence of benzoic acid (Student's t test $\alpha = 1\%$). 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 HK displayed a substantial $\rho_{mRNA,V_{max}}$ (0.45) when comparing aerobic and anaerobic conditions, indicating that transcription was for 45% regulating its V_{max} (Table 4.2, column 4). To evaluate the overall regulation of the fluxes by mRNA levels, we calculated $\rho_{mRNA,flux}$, defined by:

$$\rho_{mRNA,flux} \equiv \frac{\Delta \ln[mRNA]}{\Delta \ln flux} = \rho_h \cdot \rho_{mRNA,V_{max}} \tag{4.12}$$

In the comparison of aerobic and anaerobic cultures $\rho_{mRNA,flux}$ was at most 0.2 (HK and PFK, Table 4.2, column 6), implying that the flux was regulated for 20% by the mRNA level in these cases. In all other cases V_{max}'s and fluxes were hardly regulated by mRNA levels as reflected by low mRNA regulation coefficients (Table 4.2, columns 4 and 6).

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 (Figure 4.2 and 4.3), which resulted in relatively high errors in the $\rho_{mRNA,V_{max}}$ (Table 4.3, columns 4 and 5). The coefficients with reasonably good standard errors of the mean were low, with the exception of ENO and PDC (0.73 and 0.46 respectively).

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

4.3.4 Regulation of protein levels by translation and protein degradation

Above it was shown that, apart from a few exceptions, the mRNA levels hardly regulated the overall fluxes and enzyme capacities (V_{max}). This, however, does not exclude that specific isoenzyme concentrations are regulated by changes at the mRNA level. To examine this possibility, concentrations of the glycolytic proteins were measured by LC-MS/MS. By comparing proteins from *in vivo* ^{15}N- and ^{14}N-labelled aerobic and anaerobic chemostats, we could reliably identify and quantify 21 out of the 27 glycolytic proteins (Figure 4.4). 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 as compared to aerobic conditions, exceptions where: Glk1p, Fba1p, Tpi1p and Pgk1p which concentrations hardly changed. The regulation of the level of a protein by the corresponding mRNA level was quantified as follows:

$$\rho_{mRNA,protein} = \frac{\Delta \ln[mRNA]}{\Delta \ln[protein]} \tag{4.13}$$

For the large majority of the proteins the contribution of the change in corresponding mRNA level to the regulation of their concentration was below 30% (Table 4.4, $\rho_{mRNA,protein} < 0.3$). This included proteins displaying 6-fold changes in concentration between aerobic and anaerobic chemostats (*i.e.* Pgi1p, Pfk1p, and Pdc1p). Proteins that were only detected under anaerobic conditions (Pfk2p, Pyk1p and Pdc5)

Table 4.4: Transcription and translation/degradation regulation of the concentrations of glycolytic enzymes in anaerobic versus aerobic glucose-limited chemostats. The regulation of protein concentrations by changes in mRNA levels ($\rho_{mRNA,protein}$) were calculated as indicated in Eq. (4.13). The sum nof the contributions of changes in the rate of translation and degradation ($\rho_{trans,protein} + \rho_{dd,protein}$) as calculated by subtracting $\rho_{mRNA,protein}$ from 1, *cf* Eq. (4.15).

	$\rho_{mRNA,protein}$	SEM	$\rho_{trans,protein}+$ $\rho_{dd,protein}$
Hxk1p	0.3	0.1	0.7
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	-0.2	0.27	1.2
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

and could not be used for Regulation Analysis, were 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.* Figure 4.3 and 4.4). 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.

Since 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. (4.7) and (4.8), one finds:

$$[protein] = k_{trans} \cdot [ribosome] \cdot \frac{[mRNA]}{k_{deg} + \mu} \tag{4.14}$$

and therefore:

$$1 = \underbrace{\left(\frac{\Delta \ln k_{trans}}{\Delta \ln [protein]} + \frac{\Delta \ln [ribosome]}{\Delta \ln [protein]} \right)}_{translation} + \underbrace{\frac{\Delta \ln [mRNA]}{\Delta \ln [protein]}}_{transcription} - \underbrace{\frac{\Delta \ln (\mu + k_{deg})}{\Delta \ln [protein]}}_{degradation/dilution} \equiv \rho_{trans,protein} + \rho_{mRNA,protein} + \rho_{dd,protein} \tag{4.15}$$

Since the specific growth rate and therefore the dilution of proteins is constant in the present study, it follows that the regulation of the concentration of any protein

through translation and degradation together ($\rho_{trans,protein}+\rho_{dd,protein}$) can be calculated by subtracting the regulation through mRNA concentrations from 1. The final column of Table 4.4 gives the results of this calculation and shows that the regulation of the concentration of the glycolytic proteins appears to be regulated by translation or protein degradation much more than by transcription.

4.3.5 Posttranslational regulation

We have shown that the hierarchical regulation of V_{max} did not occur at the transcription level and therefore must have occured post-transcriptionally. Changes in V_{max} could result from (i) tuning of protein concentration by translation and/or degradation, or (ii) from modification of the kinetic properties of the enzymes by post-translational modification. We have also shown that protein concentrations are regulated to a large extent by protein translation and/or degradation. In order to investigate the contribution of post-translational processes to the regulation of V_{max}, $\rho_{PT,Vmax}$ was calculated, *cf.* Eq. (4.11). This calculation requires the concentration ratio of the sum of all isoenzymes for each reaction, rather than the concentration ratio of individual isoenzymes. For all reactions catalyzed by multiple isoenzymes (PGI, ALD, TPI and PGK are catalyzed by a single isoenzyme) the relative contributions of these isoenzymes were estimated from 2D gels or from the transcript levels when the proteins were not found on the gels (not shown) and the coefficients were calculated as follows:

$$\rho_{PT,V_{max}} \equiv \frac{\Delta \ln k_{cat}}{\Delta \ln V_{max}} = 1 - \frac{\Delta \ln[protein]}{\Delta \ln V_{max}} \tag{4.16}$$

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 (GAPDH and PGM) were only marginally regulated by posttranslational processes.

4.4 Discussion

In this study we developed a method to quantitatively dissect the regulation of gene expression into contributions by each of the processes in the gene-expression cascade. Using this method, 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 are regulated by gene-expression at all - regulation by mRNA levels plays a marginal role. Rather, most of the gene-expression regulation is exerted at the level of protein synthesis and/or degradation, and possibly also at the level of posttranslational modification of enzymes. 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 biologists attach to transcriptome analysis into 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 (ter Kuile & Westerhoff, 2001) and starving yeast cells (Chapter 3), 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 (Chapter 3) 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. 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 (ter Kuile & Westerhoff, 2001, Chapter 3) 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 regulation. The new analysis considers regulation of gene expression in terms of the dependence of the translation rate on mRNA concentration, the dependence of the protein degradation rate on the protein concentration and the occurrence of posttranslational modifications that affect the specific turnover rate k_{cat}. It also accomodates the effects of changes in specific growth rate. 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 to 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 turn-over measurements in chemostat cultures of *S. cerevisiae* support this hypothesis (Pratt *et al.*, 2002). Furthermore we assumed the rate of translation to depend proportionally on the concentration of the mRNA of interest, Eq. (4.8). This may seem a very strong assumption since 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 mRNA's encoding isoenzymes were summed. This is a simplification since isoenzymes often have different kinetic characteristics and translation yield may well differ between mRNAs encoding isoenzymes. In the present study this simplification was necessary, but 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 also here a higher resolution analysis should be useful. Since the focus of this paper is on the essence of the method, we leave such further analysis for a future study.

The attempt to identify the potential regulation of V_{max} by post-translational 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-LC/MS-MS approach were more accurate (Figure 4.4). Yet, among the 12 coefficients assessing the contribution of post-translational processes to the regulation of enzyme capacities, only two could be estimated reliably (GAPDH and PGM) and they were small, implying that in these cases posttranslational modifications contributed little to V_{max} regulation. So far potential post-translational 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 2, Pyk1 and 2, Eno1 and 2 and Gpm1p (Vojtek & Fraenkel, 1990; Portela *et al.*, 2002; Rayner *et al.*, 2002; Ficarro *et al.*, 2002; Ptacek *et al.*, 2005)) 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 ($\rho_h < 0.3$), while phosphoglycerate mutase was not regulated by posttranslational modification (Table 4.2, column 8).

If V_{max} is hardly regulated by transcript levels, and for most enzymes not by post-translational activation either, we can only conclude that most of the V_{max} regulation is at the level of translation and protein degradation rates. Actually our data were accurate enough to firmly establish that the regulation of protein concentrations was mainly at the the level of translation and degradation and hardly at the mRNA level (Table 4.4). 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) (Hinnebusch, 2005; Vilela *et al.*, 1998). In *Saccharomyces cerevisiae*, Cpa1p is the only *enzyme* experimentally shown up to date to be regulated translationally (Gaba *et al.*, 2001). All the mRNAs encoding these proteins contain upstream open-reading frames that can partly or completely disable the initiation of translation (McCarthy, 1998). However, how the translation of the glycolytic proteins is regulated, is unknown and all the mechanisms that have been described previously in yeast, such as the presence of uORFs or mRNA binding proteins, should be considered (McCarthy, 1998). Even less is known about degradation of glycolytic enzymes. Although targeted degradation of enolase 2 has already been reported (Larsen *et al.*, 2001), 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 (Arava *et al.*, 2003; Shenton *et al.*, 2006; Smirnova *et al.*,

2005). 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 (Schaaff *et al.*, 1989; Smits *et al.*, 2000; Davies & Brindle, 1992). So far all 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 as compared to an immediate or exclusive focus on transcriptomics or proteomics.

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Chapter 5

The Regulation of Enzyme Rates and Pathway fluxes in Response to a Gene Deletion

In *S. cerevisiae* the *HXK2* gene, which encodes the glycolytic enzyme hexokinase II, is involved in the regulatory mechanism known as "glucose repression". Its deletion leads to fully respiratory growth at high glucose concentrations where the wild type ferments profusely. Here we describe that deletion of the *HXK2* gene resulted in a 75% reduction in fermentative capacity, *i.e.* the specific rate of CO_2 production under glucose excess and anaerobic conditions. Using Regulation Analysis we found that the fluxes through most glycolytic and fermentative enzymes were regulated cooperatively by changes in their capacities (V_{max}) and by changes in the way they interacted with the rest of metabolism. Glucose transport and phosphofructokinase were regulated purely at the metabolic level.

The reduction of fermentative capacity in the mutant was accompanied by a remarkable resilience of the remaining capacity to nutrient starvation. After starvation, the fermentative capacity of the *hxk2*Δ mutant was similar to that of the wild type. Based on our results and previous reports, we suggest an inverse correlation between glucose repression and the resilience of fermentative capacity towards nutrient starvation.

5.1 Introduction

Glucose-limited cultures of the yeast *Saccharomyces cerevisiae* show different physiological characteristics and gene expression profiles as compared to cultures grown at

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glucose-excess. While glucose-limited cultures are characterized by a fully respiratory metabolism, glucose-excess cultures show a mixed respiro-fermentative metabolism (*e.g.* van Hoek *et al.*, 2000). Glucose-repressed genes include genes encoding proteins involved in respiration, gluconeogenesis, the glyoxylate cycle, high-affinity glucose transport and the utilization of alternative carbon sources, as well as a large group of stress-response-element (STRE) controlled genes. On the other hand, the genes of some glycolytic enzymes are induced by high glucose concentrations (reviewed in Rolland *et al.*, 2002).

In order to achieve a high biomass yield during bakers' yeast production, a completely respiratory metabolism is required. Fermentation (*i.e.* ethanol production) is avoided during the production phase by ensuring a high aeration rate and a low glucose influx. At the same time, fermentative *capacity* is an important quality parameter in the bakers' yeast industry. It is defined as the specific rate of carbon dioxide production under sugar-excess and anaerobic conditions (van Hoek *et al.*, 1998b). Cells harvested from glucose-limited cultures have a lower fermentative capacity than cells grown under glucose excess conditions (van Hoek *et al.*, 2000, 1998b). Fermentative capacity has been reported to decrease in response to nutrient starvation (Nilsson *et al.*, 2001b). The severity of this detrimental effect depended on the type of starvation (*e.g.* starvation for nitrogen versus carbon) and on the physiological state of the cultures prior to starvation. Post-diauxic shift cultures respiring ethanol preserved better their fermentative capacity than cultures respiro-fermenting glucose when challenged with nutrient starvation (Nilsson *et al.*, 2001a,b). Nutrient starvation is a relevant phenomenon for the bakers' yeast industry, since at the final stages of production and during storage, cells are starved. In general, nutrient starvation is perhaps one of the most common stresses experienced by microorganisms.

Many of the adaptations to glucose excess conditions are regulated through the "main glucose repression pathway". This signaling pathway senses the concentration of extracellular glucose and transmits this information to the transcription apparatus. Deletion of the *HXK2* gene, which encodes the glucose phosphorylating enzyme hexokinase II, alleviated glucose repression (Zimmermann & Scheel, 1977; Entian & Zimmermann, 1980; Michels & Romanowski, 1980) as evidenced by fully respiratory growth at high glucose concentrations (Diderich *et al.*, 2001), co-consumption of glucose with other sugars (Raamsdonk *et al.*, 2001), derepression of high affinity hexose transporters (Petit *et al.*, 2000) and increased plasma-membrane H^+-ATPase activity (Diderich *et al.*, 2001).

Although many components of the main glucose repression pathway are known and a sensing role of hexokinase II has been proposed, the mechanism through which hexokinase II triggers glucose repression is not fully understood. Although early studies suggested a correlation between glucose phosphorilating capacity and glucose repression (Ma *et al.*, 1989; Rose *et al.*, 1991), specific point mutations in the *HXK2* gene had differential effects on phosphorylating capacity and glucose repression (Hohmann *et al.*, 1999; Kraakman *et al.*, 1999). Deletion of *HXK1*, encoding hexokinase I, in an *hxk2*Δ mutant further alleviated glucose repression while its overexpression restored it to some extent. However, overexpression of *GLK1*, encoding glucokinase (which also catalyzes glucose phosphorylation) in a *hxk2*Δ mutant, had no effect (Rose *et al.*, 1991). Evidently, glucose-phosphorylation capacity is not the sole determinant of glu-

cose repression. In addition, hexokinase II was reported to reside partly in the nucleus (Randez-Gil *et al.*, 1998). This nuclear localization was shown to depend upon Mig1 (Ahuatzi *et al.*, 2004), a transcription factor responsible for the repression of many glucose-repressible genes (DeVit & Johnston, 1999; Ostling & Ronne, 1998). These findings led to the suggestion that hexokinase II forms a repressor complex with Mig1 that is located in the nucleus during growth on glucose (Ahuatzi *et al.*, 2004).

Most studies about the respiration-fermentation switch of yeast focus on the transcriptional regulation of the respiratory pathway. However, recent studies suggest that in yeast, gene-expression and in particular transcription correlates poorly with glycolytic enzyme capacities and fluxes (Daran-Lapujade *et al.*, 2004) and is often a minor component of the local regulation of glycolytic enzyme rates (Chapters 3 and 4). These studies show that metabolic regulation of enzyme rates was just as or even more important than their regulation by enzyme capacity changes. In the present study, we investigated the alleviation of glucose repression by the deletion of *HXK2* from the point of view of the regulation of glycolytic and fermentative fluxes. Rather than focusing on transcriptional regulation alone, we investigated the extent to which the rates of glycolytic and fermentative enzymes were regulated by changes in enzyme capacities (brought about by the hierarchical cascade of gene-expression) and the extent to which they were regulated through metabolic interactions. To this end we used Regulation Analysis (ter Kuile & Westerhoff, 2001). Its idea is as follows. Because enzymes are catalysts (and not substrates), enzyme rate equations are usually of the form:

$$v = f(e) \cdot g(\mathbf{x}, \mathbf{k}) \tag{5.1}$$

in which v is the rate, f is a function of e, which is the concentration of the enzyme catalyzing the reaction and g is a function of $\mathbf{x}$ and $\mathbf{k}$, in which $\mathbf{x}$ is a vector of concentrations of substrates, products and other metabolic effectors, and $\mathbf{k}$ is a vector of constants parametrizing the strength with which the enzymes interact with their substrates, products and allosteric effectors. The important characteristic of the above equation is that f does not depend upon $\mathbf{x}$ and $\mathbf{k}$, and g does not depend upon e. $f(e)$ describes the dependency of the rate upon the enzyme concentration and can be taken to equal V_{max}. Alterations of $g(\mathbf{x}, \mathbf{k})$ are regulated through the interaction of the enzyme with the rest of metabolism. Alterations of f reflect the regulation of the capacity of the enzyme of interest, brought about exclusively through gene-expression. Stable covalent modification of the enzyme also falls in this category. The dissection and quantification of f and g is achieved by projecting Eq. (5.1) into logarithmic space, considering a change between two steady states, and dividing both sides of the equation by the relative change in steady-state flux J. Since at steady state the flux J equals the enzyme rate v, this results in:

$$1 = \frac{\Delta \ln f(e)}{\Delta \ln J} + \frac{\Delta \ln g(\mathbf{x}, \mathbf{k})}{\Delta \ln J} = \rho_h + \rho_m \tag{5.2}$$

Here ρ_h is the "hierarchical regulation coefficient", quantifying the relative contribution of changes in enzyme capacity (V_{max}) to the regulation of the enzyme's flux. ρ_h is the "metabolic regulation coefficient", quantifying the relative contribution of changes in the interaction of the enzyme with the rest of metabolism to the regulation of the enzyme's flux. For a more elaborate description and discussion of

the method see Chapter 3. The term "hierarchical regulation coefficient" was introduced by ter Kuile & Westerhoff (2001), because the V_{max} depends on the complete gene-expression cascade of transcription, translation, posttranslational modification, and mRNA and protein degradation. The two regulation coefficients sum up to one (summation theorem for the regulation of flux) implying that determination of one coefficient will yield the other automatically (ter Kuile & Westerhoff, 2001, Chapter 2). In practice the hierarchical regulation coefficient is more readily determined, since $f(e)$ usually can be taken to equal V_{max}, and changes in the V_{max} as well as in the flux J through the enzyme can often be measured or estimated.

In this study we investigate the regulation of the glycolytic and fermentative capacity upon deletion of *HXK2*. To our knowledge this is the first study in which the anaerobic metabolism of this mutant is considered. Using Regulation Analysis, we will first dissect the regulation of the rates of glcolytic and fermentative enzymes into the contributions of changes in enzyme capacities (ρ_h) and the contributions of changes in the enzymes' interaction with the rest of metabolism (ρ_m) in response to the deletion of the *HXK2* gene. We shall do this for the regulation of fluxes through the individual glycolytic and fermentative enzymes. Subsequently we shall then examine how hexokinase II deletion impacts on the regulation of the fermentative capacity during starvation. We will show that the mutant exhibits a remarkable resilience towards nutrient starvation in terms of its fermentative capacity. Based on our results and previous reports, we suggest an inverse correlation between glucose repression and the resilience of fermentative capacity towards nutrient starvation. Further, the resilience of the *hxk2*Δ mutant towards nutrient starvation in combination with its higher biomass yield and the unavoidability of nutrient starvation during the industrial production and the storage of baker's yeast, makes this strain an attractive phenotype for the baker's yeast industry.

5.2 Materials and Methods

5.2.1 Growth and starvations

The growth and starvation procedures have been described in detail in Chapter 2. Briefly, *Saccharomyces cerevisiae* strains CEN-PK 113-7D (*MATa MAL2-8^c SUC2*) and KY116 (*MATa MAL2-8^c SUC hxk2*Δ*::KanMX4*) (Diderich *et al.*, 2001) were grown in well-aerated batch cultures at 30°C in defined mineral medium containing 101 mM glucose (Verduyn *et al.*, 1992) kept at pH 5.0 by automatic addition of KOH. Cells were harvested at an OD_{600} of 1.0 (exponential phase) and concentrated by centrifugation. Raamsdonk (2000) reported that the *hxk2*Δ mutant (KY116) grown on mineral medium containing 20 μg/l of biotin showed biotin deficiency in late exponential growth phase (OD_{600} of 4.0) but not earlier. Our studies were carried out on cells grown on a mineral medium containing 50 μg/l of biotin (Verduyn *et al.*, 1992) and harvested at an OD_{600} of 1.0. In these conditions, we confirmed that addition of higher concentrations of biotin did not affect the culture's growth rate (results not shown) and therefore we discarded the possibility of biotin deficiency.

For starvation experiments, the pellets were washed with equal volumes of ice-cold growth medium lacking either glucose or ammonium, and resuspended in the

corresponding medium at 30 ^{o}C to a cell density of 7.5 g/l wet weight (approximately 1g dry weight l^{-1}) at pH 6.0. The suspensions, of approximately 0.30 l, were kept in 2-liter shake flasks on a rotary shaker at 30 ^{o}C and 200 rpm without pH control for 24 hours. For the measurement of steady-state fluxes, the cells were harvested by centrifugation and resuspended in growth medium without a carbon source and kept on ice for at most 1 hour prior to measurement. Similarly, for the measurement of zero-*trans* influx of glucose, cells were harvested by centrifugation and resuspended in growth medium lacking carbon and nitrogen sources, and kept on ice for at most 1 hour prior to measurement.

5.2.2 Steady-state fluxes

For the measurement of fermentative capacity and other steady-state fluxes the cells were resuspended in medium lacking glucose at 30 C, kept anaerobic in a setup described by van Hoek *et al.* (1998b) with the modification that the headspace was flushed with N_2 instead of CO_2 as described in Chapter 2. At time zero 101 mM of glucose was added. Ethanol, glucose, glycerol, succinate, acetate and trehalose were monitored for 30 minutes by PCA extraction (Chapter 2) followed by HPLC (300mm x 7.8 mm Ion exchange column Aminex-HPX 87H (Biorad), with 22.5 mM H_2SO_4 kept at 55 ^{o}C as eluent at a flow rate of 0.5 ml min^{-1}). Glycogen was assayed according to Parrou & Francois (1997). The summed rates of production of acetate and succinate were always below 1% of the consumed glucose and are not reported. The fermentative capacity is defined as the specific rate of carbon dioxide production. Here the specific rate of ethanol production, which equals the CO_2 specific rate if the minor (*i.e.* less than 1%) production of acetate is neglected, was measured. In a control experiment it had been verified that ethanol evaporation was negligible under these conditions.

The measured extracellular fluxes were used to calculate the fluxes through each enzyme in the glycolytic and fermentative pathways. The flux through the glucose transporter (GLT) was taken as equal to the measured glucose consumption flux. The fluxes through enzymes downstream of hexokinase (HK) were calculated from the steady-state rates of ethanol and glycerol production. The fluxes through HK, glucose-6-phosphate isomerase (PGI), 6-phosphofructokinase (PFK) and aldolase (ALD) were calculated by dividing the sum of the glycerol and ethanol fluxes by two. The flux through triose-phosphate isomerase (TPI) was calculated by subtracting the rate of glycerol production from the flux through the previous block (HK through ALD). The fluxes through glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and the enzymes downstream were taken as equal to the measured ethanol flux. The consumed carbon matched the produced carbon within experimental error (Figure 5.1) and their means differed by 5% and 0.5% in the wild type and *hxk2*Δ mutant, respectively. Our calculation of fluxes through individual enzymes implicitly assumes that these small gaps in the carbon balances are filled by synthesis or mobilization of storage carbohydrates. Such an assumption is justified since the measured glycogen flux was small, but inherently difficult to quantify accurately due to uncertainties of extraction and calibration.

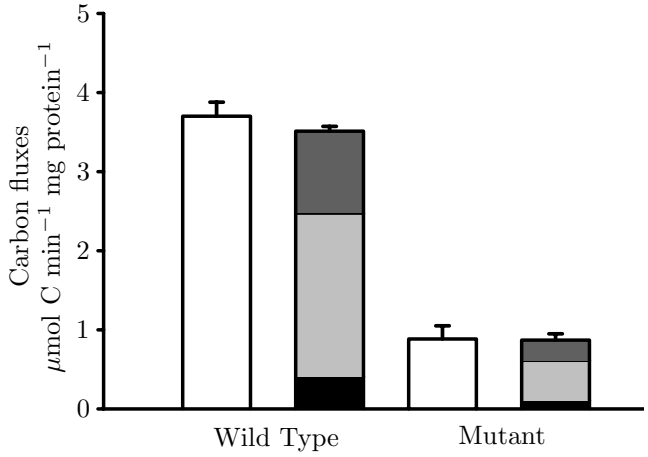

Figure 5.1: Carbon-flux balances in the wild type and *hxk2*Δ mutant. Carbon fluxes for each strain are represented with two columns: one depicting the consumed carbon (open column) and the other the produced carbon (dark shaded areas). Columns are divided into fluxes: glucose (open columns), glycerol (black areas), ethanol (light shaded areas), and CO_2 (calculated from the production of ethanol) (dark shaded areas). Other measured fluxes were negligible (see text). Error bars represent the standard error of the mean of the sum of consumed or produced carbon fluxes of four independent experiments carried out with different batches of cells. The carbon-flux balance of the wild type has been published previously (Chapter 3).

5.2.3 Zero-*trans* influx of glucose

Zero-*trans* influx of ^{14}C radiolabelled glucose was determined in a 5 second uptake assay at 30 C according to Walsh *et al.* (1994), with the modifications introduced in Chapter 2. 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.).

5.2.4 Enzyme activity measurements

Enzyme extractions and activity assays were performed as described by van Hoek *et al.* (1998b). Enzyme activities (V_{max}) were measured in freshly prepared extracts through NAD(P)H-linked assays, using a COBAS BIO (Roche, Basel) automated analyzer for spectroscopic measurements. All enzyme assays were performed with four concentrations of cell extract to confirm that reaction rates were proportional to the amount of cell extract added.

5.2.5 Regulation Analysis

Hierarchical regulation coefficients (ρ_h) to quantify the local flux regulation of glycolytic and fermentative enzymes (ter Kuile & Westerhoff, 2001, Chapter 3) in re-

sponse to deletion of the *HXK2* gene were calculated as follows:

$$\rho_h = \frac{\ln V_{max,Mutant} - \ln V_{max,WildType}}{\ln J_{Mutant} - \ln J_{WildType}} \tag{5.3}$$

in which the J refers to the *in vivo* flux through the enzyme (see section *Steady-state fluxes*) and the subscripts *Mutant* and *WildType* refer to the *hxk2*Δ mutant and the wild type strain, respectively. We performed at least three independent measurements of the V_{max}-values for each of the glycolytic and fermentative enzymes in the wild type and *hxk2*Δ mutant. The V_{max}-values were translated into logarithmic space and the mean and standard deviation (SD) of $\ln V_{max}$ were computed. For each enzyme, the mean $\ln V_{max}$-value of the wild type was subtracted from that of the mutant yielding the numerator of Eq. (5.3). The SD of the numerator of Eq. (5.3) was computed as the square root of the sum of variances of the wild type and mutant $\ln V_{max}$-values. The denominator of Eq. (5.3) and its standard deviation were computed similarly, based on four independent determinations of the flux through the enzyme of interest (see above). The ratio of the numerator and denominator of Eq. (5.3) equals the hierarchical regulation coefficient. The metabolic regulation coefficient was then calculated from $\rho_m = 1 - \rho_h$. The SD of the hierarchical regulation coefficient was calculated by multiplying ρ_h and the square root of the sum of squared coefficients of variations ($C_v = \sigma/\mu$) of the numerator and denominator. The standard error of the mean (SEM) of ρ_h was computed by dividing the SD by the square root of 3. Note that ρ_h and ρ_m share the same SD in view of the summation theorem for the regulation of flux.

5.3 Results

5.3.1 The fermentative capacity is decreased in the *hxk2*Δ mutant, but stable during starvation

We first measured the overall steady-state fluxes of glucose, ethanol, glycerol, acetate, succinate, glycogen and trehalose under standardized conditions in the mutant and the wild type. To this end, *S. cerevisiae* strains CEN.PK 113-7D and the *hxk2*Δ mutant (KY116) were grown in well-aerated and pH-controlled batch cultures. In each starvation experiment, an aliquot of cells was harvested during exponential growth and split in three parts. One part (referred to as "unstarved") was washed and transferred to an anaerobic vessel with a fresh and complete medium with excess of glucose (101 mM). This condition was meant to mimic the situation of bakers' yeast in dough (van Hoek *et al.*, 1998b). The above-mentioned fluxes were then measured over a period of 30 minutes. The other two batches of cells were washed and transferred to the same fresh medium, except that it lacked either ammonium ("nitrogen-starved cells") or glucose ("carbon-starved cells"). After 24 hours the starved cells were harvested and the fluxes were measured in a complete medium, in the same way as was done for the unstarved cells.

Figure 5.1 depicts the measured carbon fluxes for the unstarved cultures of the wild type and mutant strains. In both strains glucose was converted predominantly

Table 5.1: Fermentative capacities of the wild type and *hxk2*Δ mutant. Fermentative capacities (measured as the specific rate of ethanol production under glucose-excess and anaerobic conditions) of both strains in unstarved, nitrogen- and carbon-starved cultures are presented in mmol min^{-1} g^{-1} protein or mmol min^{-1} g^{-1} dry weight. Errors represent standard errors of the mean of four independent experiments carried out on different batches of cells.

	Wild Type		Mutant	
	FC per unit protein	FC per unit DW	FC per unit protein	FC per unit DW
Unstarved	1.04 ± 0.03	0.392 ± 0.013	0.25 ± 0.04	0.081 ± 0.016
Nitrogen starved	0.49 ± 0.05	0.072 ± 0.003	0.36 ± 0.04	0.058 ± 0.005
Carbon starved	0.33 ± 0.05	0.094 ± 0.01	0.23 ± 0.02	0.072 ± 0.005

to ethanol, glycerol and CO_2. The production fluxes of acetate and succinate and the mobilization of storage carbohydrates were always below 1% of the rate of glucose consumption (not shown). The produced carbon could be accounted for by the consumed carbon within experimental error. In the unstarved *hxk2*Δ mutant the glucose consumption flux was decreased by 75% as compared to the flux in the unstarved wild type, and this was reflected in a proportional decrease of the production fluxes of ethanol, glycerol and CO_2.

Table 5.1 shows the fermentative capacities of the wild type and mutant strains in the unstarved and starved cultures. The fermentative capacity is defined as the specific rate of CO_2 production under the dough-like conditions specified above. Here it was measured as the specific ethanol flux, which should equal the CO_2 flux under anaerobic conditions if we neglect the small acetate production. Deletion of the *HXK2* gene resulted in a 75% reduction of the fermentative capacity. The responses of the fermentative capacities of the wild type and the *hxk2* null mutant to nutrient starvation were very different. In the wild type, both types of nutrient starvation led to a substantial loss of fermentative capacity (between 50 and 70%), as was shown before (Chapter 3), while the *hxk2*Δ mutant showed no loss of fermentative capacity during 24 hours of deprivation of either carbon or nitrogen. When expressed per unit protein, the fermentative capacity of nitrogen starved cultures was even slightly increased in the mutant. The latter was not observed when fluxes were expressed per unit dry weight, since the cells accumulated carbohydrates and increased their dry-weight-to-protein ratio.

Like the wild type (Chapter 3), the mutant accumulated storage carbohydrates during nitrogen starvation and mobilized this pool during the fermentative capacity assay (results not shown). Indeed, after nitrogen starvation, the mean rate of ethanol production in the fermentative capacity assay (0.36 μmol min^{-1} mg $protein^{-1}$ –Table 5.1) exceeded the mean glucose influx (*i.e.* two times 0.15 μmol min^{-1} mg $protein^{-1}$) and even the glucose transport capacity (*i.e.* two times 0.17 μmol min^{-1} mg $protein^{-1}$).

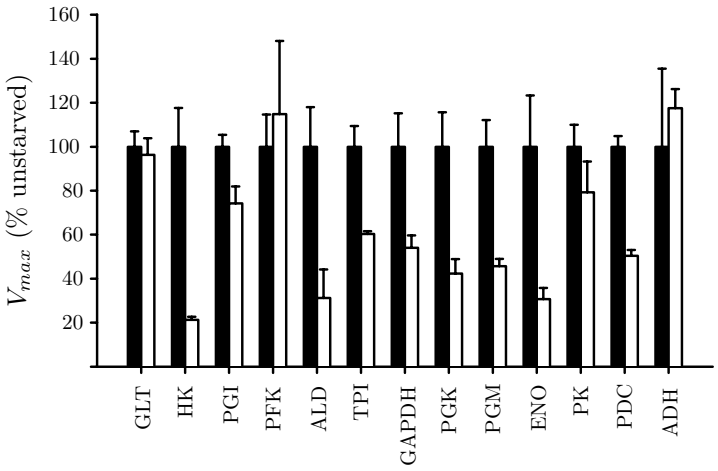

Figure 5.2: *In vitro* maximum activities of glycolytic and fermentative enzymes. The *in vitro* determined V_{max}-values of the wild type (closed columns) are compared to those of the *hxk2*Δ mutant (open columns). Error bars represent the standard errors of the mean of four (wild type) and three (mutant) independent enzyme activity measurements carried out on different batches of cells. Error bars of the GLT represent the standard errors of the mean of two independent experiments carried out on different batches of cells.

5.3.2 Enzyme activities

Deletion of the *HXK2* gene resulted in decreased fluxes through the glycolytic and fermentative enzymes. In order to investigate whether these flux reductions could be understood in terms of changes in the activities of the glucokinase and hexokinases and/or of the other enzymes in the glycolytic and fermentative pathways, we measured the maximum enzyme activities (V_{max}) in extracts from unstarved cultures of the wild type and the mutant. The absolute values are shown in Figure 5.2. Deletion of the *HXK2* gene resulted in an 80% reduction of hexokinase capacity (V_{max}), consistent with earlier results (Diderich *et al.*, 2001). This is a surprisingly strong reduction, since *HXK1* has been shown to be upregulated substantially at the mRNA level in a *hxk2*Δ mutant (Lin *et al.*, 2002). It seems that the expression of the *HXK1* is gene is regulated also by posttranscriptional mechanisms which counteract the strong transcriptional regulation. This reduction was accompanied by significant reductions in the V_{max}-values of PGI, ALD, TPI, PGK, PGM and PDC (Student's t test $\alpha = 5\%$). The reduction of PDC activity was already reported by Diderich *et al.* (2001). None of the enzyme activities was increased.

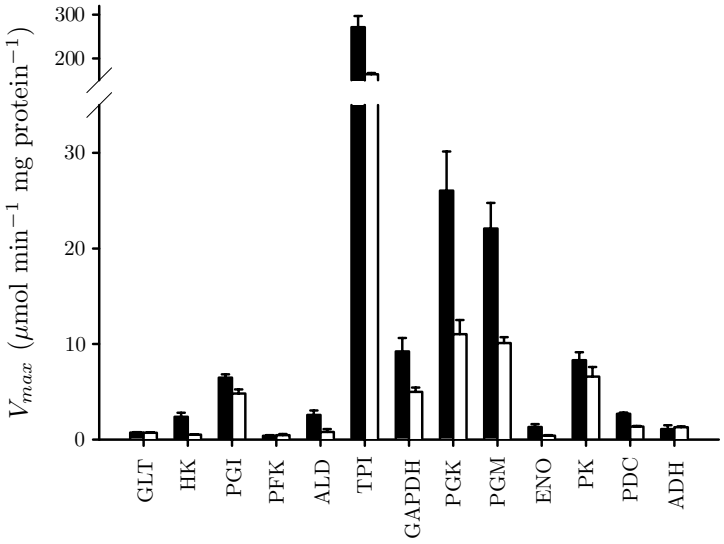

Figure 5.3: V_{max}-values of the *hxk2*Δ mutant as a percentage of those in unstarved *hxk2*Δ cultures. Unstarved (black columns), nitrogen-starved (diagonally striped columns), and carbon-starved (grey columns). Error bars of glycolytic and fermentative enzymes represent the percentage standard error of the mean, with respect to their corresponding unstarved mean V_{max}-value, of three independent experiments carried out on different batches of cells. Exceptions are GLT and PK for which two independent experiments were carried out.

5.3.3 Regulation Analysis

Deletion of the *HXK2* gene resulted in a reduced hexokinase activity that was accompanied by the reduction in the activities of PGI, ALD, TPI, PGK and PDC. These coincided with reduction of the rates of glycolytic and fermentative enzymes. In order to dissect to what extent the changes of V_{max} were responsible for the enzyme rate changes and to what extent these rates were rather regulated by changes in enzyme interactions with the rest of metabolism, we calculated the hierarchical (ρ_h) and metabolic (ρ_m) regulation coefficients for the comparison of the wild type and the mutant in unstarved cultures (*cf.* Introduction). These are summarized in Table 5.2. All the hierarchical regulation coefficients have values below 1, meaning that the relative changes in the steady-state rates of all glycolytic and fermentative enzymes are larger than the relative changes in enzyme capacities. The highest ρ_h value (0.9) was, not surprisingly, obtained for hexokinase of which one of the genes was deleted. Indeed the "gene-expression" regulation reported by this coefficient in this case is more than the response by the organism; it comprises the regulatory act by the experimenter. Aldolase and enolase were largely hierarchically regulated ($\rho_h = 0.8$). A number of enzymes were regulated cooperatively by enzyme capacity changes and changes in their interaction with the rest of metabolism. Notably GAPDH, PGK, PGM and PDC were regulated with almost equal contributions of enzyme capacity changes and changes in the way enzymes interacted with the rest of metabolism. One of the hi-

Table 5.2: Hierarchical and metabolic regulation coefficients of the regulation ensuing from the deletion of the *HXK2* gene. Hierarchical (ρ_h) and metabolic (ρ_h) regulation coefficients were calculated as described in the Materials and Methods section. Errors were quantified with standard errors of the mean (SEM) calculated for four (wild type) and three (mutant) independent V_{max} measurements and four independent flux estimations, all performed on independent batches of cells.

	ρ_h	SEM	ρ_m
GLT	0.0	0.1	1
HK	0.9	0.2	0.1
PGI	0.2	0.1	0.8
PFK	0.0	0.2	1
ALD	0.8	0.4	0.2
TPI	0.3	0.1	0.7
GAPDH	0.4	0.2	0.6
PGK	0.6	0.2	0.4
PGM	0.5	0.1	0.5
ENO	0.8	0.2	0.2
PK	0.2	0.2	0.8
PDC	0.5	0.1	0.5
ADH	-0.2	0.3	1.2

erarchical regulation coefficients was negative (ADH), indicating that the flux and the enzyme activity changed in opposite direction. However, the value of this coefficient did not differ significantly from zero and we interpreted this as a predominantly metabolic regulation. Glucose transport and PFK were regulated exclusively at the metabolic level ($\rho_m = 1.0$), without any significant hierarchical regulation. Also PGI and PK were predominantly regulated by metabolism ($\rho_m = 0.8$). Significant antagonistic regulation (*cf.* Chapter 3) was not observed (the negative but statistically insignificant ρ_h of ADH not withstanding).

5.4 Discussion

We investigated how the rates of the glycolytic and fermentative enzymes was regulated upon deletion of *hxk2* and upon starvation. This is a part of a larger research programme in which we quantitatively investigate the interplay of metabolism and gene expression, with glycolysis in bakers' yeast as our model system. Previous studies focused on the regulation of flux in response to environmental perturbations (Chapters 3 and 4). These studies showed that existing paradigms of multi- or single-site regulation did not apply to the regulation of yeast glycolysis. In multisite modulation all V_{max} values would be regulated proportionally, leading to metabolite homeostasis upon large flux changes (Fell & Thomas, 1995). Single-site modulation assumes that only one rate-controlling enzyme is regulated hierarchically.

The present study is the first example in which Regulation Analysis was applied to regulation in response to a gene deletion. *HXK2* is a special gene in that it en-

codes a protein that acts both as an enzyme catalyzing a glycolytic reaction and as a component of the main glucose-repression pathway. Upon deletion of *HXK2* we found a broad range of combinations of metabolic and hierarchical regulation, from purely metabolic ($\rho_h = 0$) to purely hierarchical ($\rho_h = 1$). The majority of hierarchical regulation coefficients ranged between 0 and 1. This means that the regulation of the fluxes through those enzymes was brought about cooperatively by changes in the V_{max}'s of the enzymes and their interactions with the rest of metabolism (Chapter 2). The finding that another type of perturbation again resulted in a broad range of combinations of metabolic and hierarchical regulation, corroborates our earlier conclusion that the regulation of glycolytic fluxes in bakers' yeast is variegated and may not be driven by single drives or constraints as suggested by single- or multi-site regulation hypotheses.

It is known that several genes encoding glycolytic enzymes are under the transcriptional control of the GCR1/GCR2/RAP2 system (Chambers *et al.*, 1995). The deletion of *gcr1* and/or *gcr2* results in decreased *in vitro* activities of the majority of glycolytic enzymes in cells that are grown on glucose but not in those grown on non-fermentable carbon sources (Clifton & Fraenkel, 1981; Uemura & Fraenkel, 1990). The fact that in our study the glycolytic enzyme activities were regulated differentially (Figure 5.2), some remaining constant and others decreasing to variable extents in the mutant, suggests that the glycolytic genes are not simply co-regulated at the transcriptional level. Moreover, if we compare the regulation of glycolysis upon different perturbations, such as the transition from aerobic to anaerobic conditions (Chapter 4) or the starvation of the wild type for nitrogen or carbon (Chapter 3), it becomes clear that the distribution of regulation between metabolism and gene expression differs between conditions. This further suggests that the glycolytic genes are regulated more subtly by other mechanisms on top of or interacting with the GCR1/GCR2/RAP2 system. For instance, the gene *SGC1* has been described as a suppressor of the GCR system (Sato *et al.*, 1999). Furthermore, regulation of mRNA stability has been implicated in glucose repression and could therefore be involved in the response of the *hxk2*Δ mutant (de la Cruz *et al.*, 2002). The interactions between the main glucose repression pathway in which Hxk2p functions, the GCR1/GCR2/RAP2 system, and possible other mechanisms such as regulation of mRNA stability, require further study, and in view of the above, a quantitative study.

At high sugar concentrations the *hxk2*Δ strain has an almost completely respiratory metabolism (Diderich *et al.*, 2001) and therefore a much higher biomass yield than the wild type. This would alleviate the need for a restricted glucose influx and thorough mixing of glucose in the bakers' yeast production phase. The finding that the fermentative capacity of the mutant was much lower than that of the wild type at first sight seemed to disqualify the mutant for application purposes. However, also the wild type has a much lower fermentative capacity when grown under respiratory conditions. Moreover, after starvation, an integral part of the overall bakers' yeast production process, the wild type and the mutant had similar fermentative capacities. This makes the *hxk2*Δ an interesting starting point for growing yeast at faster rates in (fed-)batch cultures and with more efficient substrate utilization.

The constancy of the fermentative capacity in the *hxk2*Δ mutant during both types of starvation in spite of a strong decrease in its glucose transport capacity to-

gether with the observation that among the glycolytic and fermentative enzymes the glucose transport activity was the only one that decreased significantly, suggest that the glucose transporter had no control on the fermentative capacity in the mutant. Quantification of the control of the glucose transporter upon the glycolytic flux has been attained through titration of the glucose transport capacity by addition of maltose, a competitive inhibitor of the glucose transporter (Diderich *et al.*, 1999b). The inhibition constant of maltose for the glucose transporters is between 32 and 42 mM (Reijenga *et al.*, 2005). In order to estimate the extent of the inhibition a high concentration (250 mM) of maltose would have on glucose transporter, we measured the affinity of the *hxk2*Δ mutant transporters for glucose ($K_m = 5.4$ mM) and calculated the rate of glucose transport in the presence and absence of maltose with an external glucose concentration of 101 mM, neglecting the intracellular glucose concentration. These calculations yielded the estimation that 250 mM of maltose should exert a 23-28% inhibition of the glucose transporter in the mutant. However, the standard deviation of the ethanol flux in the unstarved *hxk2*Δ mutant is 32% of its mean, which implies that if the glucose transporter would have a control coefficient of 1 and the ethanol production would decrease in proportion to the decreased glucose transport rate, the change in ethanol flux would not be distinguishable from statistical variation. Thus titration with maltose is an unviable option for the assessment of the control of the glucose transporter upon ethanol production in the *hxk2*Δ mutant in our experimental setup.

The resilience of the *hxk2* null mutant towards nutrient starvation is consistent with results obtained for other respiratory yeast cultures. Nilsson *et al.* reported that post-diauxic shift cultures respiring ethanol preserved their fermentative capacity better than cells growing on glucose when challenged by nutrient starvation (Nilsson *et al.*, 2001b). Analogously, the fermentative capacity of fully respiratory, glucose-limited chemostat cultures of *S. cerevisiae* at low dilution rates was also resilient to nitrogen or carbon starvation (Van Eunen and Bakker, unpublished). These findings together suggest that the stability of the fermentative capacity upon nutrient starvation is inversely correlated with glucose repression.

Acknowledgements

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Chapter 6

General Discussion

This thesis is concerned with the study of the regulatory processes involved in the adaptations of metabolic systems to environmental and genetic changes. In the first Chapter of this thesis it was argued that the study of regulation is an endeavor unique to biology and that our understanding of regulatory processes is hindered by the lack of a precise definition of the term regulation and of appropriate methodologies to describe regulatory process in an unambiguous and quantitative manner. The chapters in this thesis report the implementation, evaluation and further development of a method that enables a quantitative description of the regulation of enzyme rates and their catalytic capacites. This method was implemented, tested and elaborated in a series of investigations upon the regulation of *Saccharomyces cerevisiae*'s glycolysis to nutrient starvation, oxygen deprivation, increased free-energy dissipation by addition of benzoic acid, and deletion of the gene *HXK2* encoding hexokinase II. The experimental findings and analyses yielded new insights in to the complexity of the regulation of metabolic fluxes and the catalytic capacities of the enzymes catalyzing their reactions.

The study of regulation requires a precise definition of the term and the terminology to describe regulatory processes unambiguously. As outlined in the General Introduction, several efforts to convey a precise and quantitative definition of metabolic regulation were based on Metabolic Control Analysis (MCA) and addressed the study of the regulation of enzyme rates by the metabolites that affected them (Sauro, 1989) or the regulation of metabolic system properties by changes in external or internal metabolite concentrations (Westerhoff & Chen, 1984; Hofmeyr & Cornish-Bowden, 1991; Kahn & Westerhoff, 1993; Hofmeyr, 1995), all disregarding the regulation by gene-expression. These studies were explorations of the MCA theoretical framework without experimental investigation of real biological systems. In contrast, Regulation Analysis was devised to aid the *experimental* investigation of the regulation of enzyme-catalyzed rates by gene-expression and metabolic interactions.

It is important to stress the descriptive nature of Regulation Analysis and its association with and dependence on experimentation. In contrast to MCA-based approaches, Regulation Analysis does not require a complete kinetic characterization of the metabolic network but relies on direct measurement of enzyme rates and amounts.

By being extricated from the need of a complete kinetic description of the metabolic system, Regulation Analysis is restricted to the description of the local regulation of enzyme rates and is therefore devoid of predictive capacity. Its association with experiments, on the other hand, makes it more appropriate for the study of biological systems for which a complete kinetic characterization is not yet available.

In this chapter, Regulation Analysis is discussed in terms of its scope, the assumptions used in its derivation and the interpretation of its numerical outcomes. This methodological discussion is followed by a discussion of the experimental results reported in this thesis, with an emphasis on the quantitative description provided by Regulation Analysis.

6.1 Hierarchical and Metabolic Regulation of Enzyme Rates

Throughout this thesis Regulation Analysis has been used to dissect the regulation of fluxes through individual metabolic enzymes into its "hierarchical" and "metabolic" components. Ter Kuile and Westerhoff defined hierarchical regulation as the component of regulation that acts via the gene-expression cascade and metabolic regulation as the component that affects the way in which enzymes interact with the rest of metabolism (ter Kuile, 1996). The dissection of the hierarchical and metabolic components is possible thanks to the fact that enzymes are catalysts, of which the amounts are unchanged by the reaction which they catalyze. Therefore, enzyme-catalyzed rates are most often proportional to the amount of enzymes present, *cf.* Eqs. (1.37) – (1.39). Importantly, the amount of enzyme present is not a function of the concentrations of metabolites that interact with that enzyme and the interactions of the enzyme with these metabolites is not a function of the amount of enzyme. This cross-independence between the amount of catalyst and the way it interacts with the rest of the system permits the dissection of the contributions of these two factors to the regulation of the enzyme-catalyzed rates for arbitrarily large changes in the amount of enzyme. It is this feature of Regulation Analysis that distinguishes it as a method suitable for the experimental investigation of the regulation of enzyme-catalyzed rates.

Regulation Analysis requires the experimental determination of the amount of enzyme catalyzing the reaction studied. Because *changes* in enzyme amounts rather than absolute values are considered in Regulation Analysis accuracy is of utmost importance. V_{max} measurements are highly specific and their precision and reproducibilitly is far better than direct measurement of enzyme amounts by state-of-the-art proteomics. Following Ter Kuile and Westerhoff's original presentation of the method, in this thesis relative changes in enzyme capacities (V_{max}) have been used as a measure of relative changes in enzyme amounts and used for the calculation of hierarchical regulation coefficients. However, the subtlety with which real cells regulate fluxes through individual metabolic steps and a number experimental limitations warrant examination of the appropriateness of the use of V_{max} changes as a measure of changes in enzyme amount and the interpretation of hierarchical regulation coefficients calculated using V_{max} measurements.

6.1.1 Enzyme capacities (V_{max}) as a measure of enzyme amounts

Relative changes in V_{max} are exactly equal to relative changes in enzyme amounts if the enzyme's k_{cat} does not change between the conditions considered. However, if k_{cat} changes between the conditions considered the relative change in V_{max} may be very different from the relative changes in enzyme amounts.

The k_{cat} of an enzyme may change due to covalent modifications. Chapter 3 discussed the modulation of V_{max} by phosphorylation during transfer of starved cultures to a complete medium as it occurs during the fermentative capacity assay. In a subsequent pilot experiment, the V_{max} of glycolytic and fermentative enzymes was measured in starved and unstarved cultures harvested 15 minutes after their transfer to complete medium. For each condition two batches of cells were taken for V_{max} measurements. In one batch, enzyme extraction and the V_{max} measurements were performed in the presence of phosphatase inhibitors, in the other batch enzymes were extracted and their V_{max} was measured in the absence of phosphatase inhibitors. The differences in V_{max} observed between the two extraction procedures were not beyond what was expected on the bases of statistical variation with the exception of pyruvate kinase, which has been reported to be activated through phosphorylation by protein kinase A (Portela *et al.*, 2002). Figure 6.1 shows the V_{max} measurements of pyruvate kinase extracted and assayed in the presence or absence of phosphatase inhibitors when samples were harvested 15 minutes after the transfer of cells to complete medium. The V_{max} value of pyruvate kinase in nitrogen or carbon starved, but not in unstarved, cultures was many times higher when dephosphorylation was prevented by addition of phosphatase inhibitors. The deviation of relative changes in V_{max} from that of enzyme amounts is apparent in both types of starvation where the measured pyruvate kinase V_{max} change suggested a decrease in enzyme amounts when assayed in the absence of phosphatase inhibitors and an increase when assayed in their presence. While among *S. cerevisiae*'s glycolytic and fermentative enzymes, activity modulation by phosphorylation has only been reported for pyruvate kinase, other covalent modifications may still affect the catalytic constants of other glycolytic enzymes. The occurrence of posttranslational modifications affecting the catalytic constants of triosephosphate isomerase and phosphoglycerate kinase is suggested by the protein measurements reported in Chapter 4, although the standard errors of the mean were too large for the experiments to be decisive.

6.1.2 Differential expression of isoenzymes

One metabolic reaction may be catalyzed by different proteins which are products of different genes. Different enzymes that catalyze the same reaction are called isoenzymes. In fact, the majority of reactions in the glycolytic and fermentative pathways in *S. cerevisiae* are catalyzed by two or more isoenzymes. Since isoenzymes are different proteins they may have different catalytic properties. Their catalytic constants k_{cat} may differ as well as their affinities for substrates, products and other metabolic effectors. Isoenzymes may be expressed differentially in such a way that the apparent catalytic properties of the isoenzyme pool will vary between conditions. This feature of modulation of apparent catalytic properties of an isoenzyme pool is particularly apparent in yeast glucose transport, where the apparent affinity of the transporters

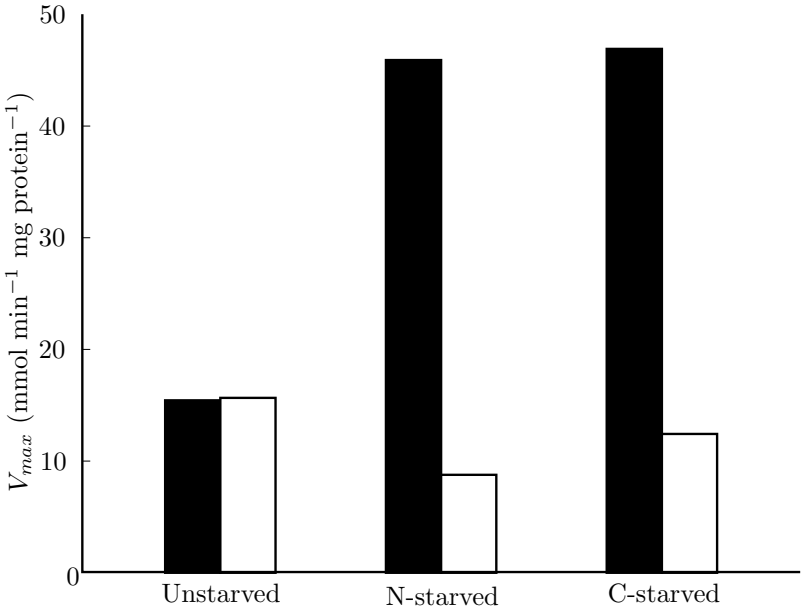

Figure 6.1: V_{max}-values of pyruvate kinase assayed in the presence or absence of phosphatase inhibitors. V_{max}-values in $\mu mol\, min^{-1}\, mg\, protein^{-1}$ are plotted. The measurements were made in an experiment where the cells were harvested 15 minutes after being transferred to a complete medium. For each condition, one batch of these cells was extracted and assayed in the presence of phosphatase inhibitors (black bars) and another batch in absence of the latter(open bars). Unstarved refers to cells harvested during the exponetial phase (OD = 0.1) of a batch culture. The starved cultures were harvested during the exponential phase of the batch culture and subsequently deprived of nitrogen (N-starved) or carbon (C-starved) for 24 hours.

for glucose is modulated through the differential expression of hexose transporters with different glucose affinities (Diderich *et al.*, 1999a). In Chapter 2, it was discussed that differential expression of isoenzymes with different metabolite affinities modulates the interaction of the isoenzyme population with the rest of metabolism. If the hierarchical regulation coefficient is then calculated from overall V_{max} change of the isoenzyme pool, the analysis does not dissect gene-expression regulation from metabolic regulation, since the expression of genes affects both the V_{max} and the way in which the enzyme population interacts with the rest of metabolism. In such cases, the hierarchical regulation coefficient measures the regulation by V_{max} changes, which are effected exclusively by gene expression, and the metabolic regulation coefficient measures the regulation by changes in the way the enzyme population interacts with the rest of metabolism, which may be effected by both gene expression and metabolite concentration changes.

In principle it is possible to disentangle the contributions of gene expression and metabolic changes for metabolic reactions catalyzed by populations of isoenzymes with different kinetic properties. This dissection would require the direct quantification of single isoenzyme amounts and the fluxes through single isoenzymes. The

former requirement may be fulfilled, though it is technically very challenging in view of the accuracy required (*cf.* Chapter 4). However, the latter requirement, the measurement of fluxes through individual isoenzymes, is impossible to fulfill. It may, however, be possible to calculate the fluxes through individual isoenzymes provided that the kinetic characterization of each isoenzyme is complete and that the concentrations of all metabolites affecting their rates of catalysis are known with sufficient precision.

6.1.3 Protein-protein interactions

Protein-protein interactions may affect the kinetic properties of enzymes. For instance, the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) has been shown to form monomers, trimers and tetramers all with the same specific activity when assayed in substrate excess conditions (implying that the k_{cat} of GAPDH was unaffected by the formation of oligomers), but with different specific activities in the presence of subsaturating concentrations of substrates (suggesting that the formation of oligomers affects the enzyme's metabolite affinities) (Ashmarina *et al.*, 1982). From the viewpoint of Regulation Analysis, the fact that enzyme amounts may affect both the enzyme's V_{max} capacity and the way it interacts with the rest of metabolism, disrupts the cross-independence on which the analysis is based and impedes in these cases the dissection of hierarchical and metabolic contributions to the regulation of enzyme rates.

If a protein-protein interaction would affect the k_{cat} of an enzyme, relative changes of the V_{max} relative would deviate from relative changes of the enzyme concentration. Because protein-protein interactions depend upon the concentrations of the proteins involved, those affecting the catalytic constants of the enzyme may be identified by assaying the V_{max} in samples with different dilutions. This was routinely done in the experiments reported in this thesis and modifications of catalytic constants by protein-protein associations, if any, could not be distinguished from the experimental statistical variation.

6.1.4 The interpretation of ρ_h calculated from V_{max} measurements

The subsections above have highlighted that relative changes of V_{max} may deviate from relative changes of enzyme amount due to posttranslational modifications or protein-protein interactions, and that the interaction of the enzyme with the rest of metabolism may be modulated through the differential expression of genes encoding isoenzymes. Clearly, in these cases, Regulation Analysis using V_{max} measurements does not dissect the contributions of gene-expression and metabolic interactions to the regulation of enzyme catalyzed rates. The hierarchical regulation coefficient calculated using V_{max} measurements quantifies the contribution of V_{max} changes and distinguishes it from the regulation by all changes affecting the way the enzyme interacts with the rest of metabolism. This interpretation of the hierarchical regulation coefficient was assumed throughout this thesis. It may be argued that modulations of k_{cat} can be considered as part of the gene-expression cascade and included in the hi-

erarchical component of the regulation. If it is chosen to include modulations of k_{cat} in the hierarchical regulation, then Regulation Analysis using V_{max} measurements dissects the contributions of gene-expression and metabolic interactions to the regulation of enzyme-catalyzed rates in the cases where there is no differential expression of isoenzymes. However, it cannot be discarded that k_{cat} modulations may depend on metabolite concentrations. For instance, a metabolite concentration may influence the equilibrium between oligomeric forms of the protein which may exhibit different k_{cat}'s. For example, pyruvate decarboxylase in yeast is a multimeric enzyme that may form dimers or tetramers. The specific activity of the tetramer form is much higher than that of the dimer form and the ratio of tetramers to dimers is affected by the pH. At low pH (6.2-7.5) the tetramer is more abundant than the dimer while at higher pH (7.5-8.4) the dimer is more abundant. An increase in the concentration of pyruvate is likely to be accompained by a reduction of pH and hence an increase in the concentration of the catalytically more active tetramer. Therefore, it is plausible that changes in the concentration of pyruvate can modulate the catalytic capacity of the pyruvate decarboxylase without affecting its concentration. (Koenig *et al.*, 1992).

The fact that in practice gene-expression regulation cannot always be dissected from metabolic regulation should not distract from the value of dissecting the regulation of enzyme rates into the contributions of a measured property (V_{max}) and the contributions by the many, difficult to measure, changes affecting the enzyme's interaction with the rest of metabolism.

6.2 Dissection of the Regulation of V_{max}

The standard Regulation Analysis is based on the feature that the rates of most enzyme-catalyzed reactions are directly proportional to the amount of enzyme and on the frequent cross-independence between the enzyme amount and the function describing the interactions of the enzyme with its metabolic effectors. The dissection of the contributions of the multipliers in the rate equation is achieved by transforming the equation into logarithmic space and normalizing the resulting expression. In Chapter 4, a similar method was applied in order to dissect the contributions of the various processes in the gene-expression cascade, to the regulation of enzyme capacities (V_{max}). In order to express enzyme capacities as a multiplicative function two assumptions are made. First, it is assumed that the protein concentration is in steady-state, Eq. (4.7). This assumption is likely to be warranted by the use of chemostats (but see Jansen *et al.*, 2005). Second, it is assumed that the rate of degradation of a specific protein is directly proportional to the concentration of that protein [Eq. (4.8)] or that the rate of protein degradation is negligible compared to the rate of dilution by growth. In contrast to the relation between enzyme amounts and the rate of catalysis, the protein concentration referred to here is that of the substrate rather than that of the catalyst of the degradation reaction. Therefore the assumption of direct proportionality cannot be made on the same basis as in the former case. For the rate of protein degradation, the assumption of direct proportionality is an *approximation* based on another assumption: that the protein concentration is much lower than the half-saturation constant of the proteolytic machinery for that protein. This assump-

tion of an unsaturated proteolytic machinery for a particular protein species may be appropriate if the concentration of the protein in question is indeed very low or if the proteolytic machinery is unspecific and degrades many different protein species of which the species in question constitutes but a small fraction whilst the sum of the all other protein concentrations does not change. It must be emphasized that these two assumptions of protein concentration being in steady-state and direct proportionality between protein degradation rate and protein concentrations (or that protein degradation rates are negligible compared to the dilution rate) are indispensable for the analysis. Without them it is not possible to express enzyme capacities as a multiplicative function of translation, degradation and dilution rates, and posttranslational modifications and this would impede the dissection of these contributions, *cf.* Eqs. (4.10) and (4.11). A fact that argues against the validity of the assumption of direct proportionality between protein degradation rates and *glycolytic* protein concentrations is that the latter are highly abundant proteins in yeast, constituting under some conditions up to 65% of the cytosolic proteins (Boiteux & Hess, 1981). However, single glycolytic enzymes may still constitute minorities among the total pool of proteins degraded. Elucidating whether the approximation of direct proportionality of degradation of specific proteins and the concentration of that protein is valid will require higher precision in the measurements of protein turnovers. Existing measurements do not highlight glycolytic enzymes as being degraded differently than other, less abundant proteins (Pratt *et al.*, 2002). The results of the aforementioned protein turnover measurements are compatible with a direct proportionality of the rates of degradation and the protein concentrations, but their accuracy is not sufficient to definitively rule out hyperbolic or sigmoidal dependencies.

A third assumption used in the derivation of the extension of Regulation Analysis to study the regulation of enzyme capacities is that the rate of translation of a particular mRNA is directly proportional to its concentration. This assumption is based on the same reasoning as that explained for the rate of protein degradation, but it is not essential. Without it the expression of enzyme capacities as multiplicative functions is still possible, if regulation of the rate of translation is measured directly by, for instance pulse chase experiments with labeled amino acids.

6.3 Summary and Discussion of the Experimental Findings

In the previous sections the methodological aspects of Regulation Analysis were discussed. The aim of the previous section was to clarify the interpretation of the numerical outcomes of Regulation Analysis and to raise awareness of subtleties that may confuse their interpretation. In this section, the experimental findings reported in this thesis are discussed with emphasis on the unambiguous statements that are possible when a quantitative description is provided.

6.3.1 The regulation of enzyme rates in *S. cerevisiae*'s glycolysis

In Chapter 2 it was argued that, in principle, enzyme rates could be regulated in five different modes each with a precise biochemical interpretation and associated with precise numerical values or ranges of the hierarchical regulation coefficient. It was hypothesized that enzyme rates may be regulated exclusively by V_{max} changes ($\rho_h = 1$), exclusively by changes in the way the enzyme interacts with the rest of metabolism, ($\rho_h = 0$) or by a combination of changes of both V_{max} and metabolic interactions, which could elicit the rate change synergistically ($0 < \rho_h < 1$) or have antagonistic influences that may be either dominated by the V_{max} change ($\rho_h > 1$) or by the changes in the way the enzyme interacts with the rest of metabolism ($\rho_h < 0$). Chapters 2 to 5 show that every one of the hypothesized modes of regulation is in fact realized in real cells when adapting to environmental or genetic changes.

All the experiments presented in this thesis taken together suggest that hierarchical and metabolic contributions to the regulation of enzyme-catalyzed rates depend upon the localization of the enzyme in the metabolic network and upon the environmental or genetic change challenging the system. The regulation of enzyme rates within the glycolytic pathway was shown to be variegated both in terms of different enzymes in the pathway being regulated differently in response to a particular challenge, and in terms of a particular enzyme being regulated differently when the system responded to different challenges.

The results and analyses presented in this thesis permit the unequivocal assertion that enzyme rates are locally regulated by the cell through changes in the amount of catalyst but also through changes in the way enzymes interact with the rest of the metabolic network. The latter changes may be in turn be elicited by changes in the amount of *other* enzymes in the network as well as by changes in the cell's environment (*e.g.* a substrate concentration change).

The unequal distribution of the hierarchical regulation in a single pathway suggests that enzymes play different roles in the regulation of fluxes. Those enzymes with large positive hierarchical regulation coefficients appear to "lead" the flux change while those with positive and small hierarchical regulation coefficients appear to "follow" the leading enzymes. Still other enzymes appear to actively oppose the ensuing flux change by changing their activities in opposite direction, presumably fulfilling other regulatory functions than flux changes.

An interesting observation made both when exponentially growing cultures adapted to carbon deprivation and when the wild type strain adapted to deletion of the *HXK2* gene, was that the relative change in rate through each glycolytic enzyme was larger than the measured relative change in its V_{max} value, *i.e.* all ρ_h's were smaller than 1. This implies that in all cases the rate change could not be attributed to the V_{max} change alone but must have been partly elicited by changes in the way enzymes interacted with the rest of metabolism. From the point of view of the hypothesized roles of enzymes in the regulation of pathway fluxes, it appears that the "leading" enzymes were situated outside the glycolytic pathway and that all glycolytic enzymes "followed" a foreign lead.

A similar observation was made when glucose-limited cultures adapted to anaer-

obiosis. In this case, the fluxes were measured in the cultures and a part of the flux change may be ascribed to a difference in the residual glucose concentrations between aerobic and anaerobic cultures. In Chapters 2, 3 and 5 however, fluxes were measured off-line in fresh medium with a saturating glucose concentration. *i.e.* the flux changes were ascribable to the differences in the history of the cultures and not to an environmental factor such as glucose concentration.

The variegated regulation of glycolytic enzyme rates in terms of their position in the pathway has also been observed in the eukaryotic human parasites *Trypanosoma brucei*, *Leishmania donovani* and *Trichomonas vaginalis* when grown in chemostats at various dilution rates (ter Kuile & Westerhoff, 2001), which suggests that this observation is not a peculiarity of *S. cerevisiea*'s glycolytic pathway but is a feature shared by other microorganisms. It is possible, however, that the variegated regulation of enzyme rates is a peculiarity of glycolysis, perhaps associated with being in the core of primary metabolism and its numerous interactions with the rest of the network (*cf* section 1.4 General Introduction). There are few studies in which the activities of all enzymes in a pathway and the *in vivo* rates of these enzymes have been measured when cells respond to a variety of conditions. Such studies would be necessary to investigate whether other pathways are regulated in a similar, variegated fashion as glycolysis.

6.3.2 The complexity of V_{max} regulation

In Chapter 4 it is reported that mRNA concentration changes correlate poorly with changes in enzyme amounts or capacities. Using the extended version of Regulation Analysis it is suggested that mRNA changes account for less than 50% of the regulation of enzyme amounts and capacities. The analysis, with the assumptions outlined in the previous section, predicts that the regulation of enzyme amounts is effected predominantly by changes in the rate of translation, the rate of protein degradation or both.

Since measured mRNA changes cannot account for the observed changes in enzyme capacities and amounts, it is clear that other processes in the gene-expression cascade need to be investigated quantitatively and in more detail. The analysis presented in Chapter 4 suggests as a starting point to investigate whether translation rates and protein degradation rates are directly proportional to the concentrations of the corresponding mRNA and protein. If these assumptions are supported, then the prediction that changes in the kinetics of translation and degradation of proteins are major contributors to the regulation of enzyme capacities can be tested.

6.4 Concluding Remarks and Future Directions

In the General Introduction of this thesis it was argued that important obstacles in the advancement of our understanding of regulation of metabolic systems are the complexity of biological systems, and the lack of a precise definition of regulation and of methodologies appropriate for its study. In accordance with this view, a method to study regulatory processes has been proposed. The method contrasts with approaches based on Metabolic Control Analysis in that it is extricated from the need of a detailed

description of the complexity of real metabolic systems but also in that it is limited to a description of regulation of local enzyme rates. The description of the regulation of enzyme rates using Regulation Analysis implements a view of regulation distinct from the concept of control. It focuses on the processes occurring when real cells *adapt* to different challenges, rather than on the potential response that a completely characterized system with fixed kinetic parameters may exhibit, which is what MCA based approaches focus on.

The efforts to describe regulatory processes unambiguously and quantitatively have led to the recognition of the importance of a number of aspects of enzyme-catalyzed rates that are important in the functioning of enzymes in real cells, but have been overlooked in purely theoretical approaches. At the same time, experimental limitations have obliged the refinement of the interpretation of the outcomes of the method so that it remains useful in the aid of the explanation and communication of experimental results.

At present, a theory that explains and predicts the metabolic responses of living cells to given stimuli does not exist. Such theory will have to be built by induction based on a body of observations. This thesis reports a number of experimental observations that have been organized and expressed in a quantitative and unambiguous manner using Regulation Analysis. These findings and analyses have unravelled a previously undescribed complexity of the regulation of metabolic systems. They prove that the regulation of metabolic fluxes is not governed by single drives or constraints and that the regulation of system properties may not rely on the modulation of the properties of a single component or process. Perhaps the most important contribution of the work presented in this thesis is the proposal of a strategy for the collection, organization and unambiguous expression of experimental observations on regulatory processes in metabolic systems. It is my hope that this strategy will prove of use in the assemblage of the body of observations upon which a theory of metabolic regulation can be built.

Future directions for improving our understanding of metabolic regulatory processes are suggested by the work presented in Chapter 3 where several hypotheses on the global regulation of fluxes are expounded and their predictions in terms of the local regulation of the rates of the pathway's constitutive enzymes are formulated. The aforementioned hypotheses each suggest a different drive or constraint as director of the regulation of pathway fluxes, they were formulated in such a way that their predictions could be subject to experimental verification. The falsification of these hypotheses for the regulation of the glycolytic flux in *Saccharomyces cerevisiae* led to the realization that flux regulation is exerted in a more subtle way than previously suggested. At the same time, it highlighted the need for new hypotheses upon what are the drives and constraints governing the regulation of metabolic systems. The predictions of these new hypotheses should be formulated explicitely in terms of the experimental procedures required for their falsification. Preferably the predictions should be formulated with the experimental limitations and the complexity of biological systems in mind.

Bibliography

Abeliovich, H. & Klionsky, D.J. (2001). Autophagy in yeast: mechanistic insights and physiological function. *Microbiol Mol Biol Rev*, **65**, 463–79.

Ahuatzi, D., Herrero, P., de la Cera, T. & Moreno, F. (2004). The glucose-regulated nuclear localization of hexokinase 2 in saccharomyces cerevisiae is mig1-dependent. *J Biol Chem*, **279**, 14440–6.

Andreassen, A. & Stier, T. (1953). Anaerobic nutrition of saccharomyces cerevisiae. i. ergosterol requirement for growth in a defined medium. *J Cell Comp Physiol*, **41**, 23–26.

Aon, M.A. & Cortassa, S. (1994). Metabolic control analysis of glycolysis and branching to ethanol production in chemostat cultures of saccharomyces cerevisiae under carbon, nitrogen, or phosphate limitations. *Enzyme and Microbial Technology*, **16**, 761–770.

Arava, Y., Wang, Y., Storey, J.D., Liu, C.L., Brown, P.O. & Herschlag, D. (2003). Genome-wide analysis of mrna translation profiles in saccharomyces cerevisiae. *Proc Natl Acad Sci U S A*, **100**, 3889–94.

Ashmarina, L.I., Muronetz, V.I. & Nagradova, N.K. (1982). Evidence for a change in catalytic properties of glyceraldehyde 3-phosphate dehydrogenase monomers upon their association in a tetramer. *FEBS Lett*, **144**, 43–6.

Bakker, B.M., Overkamp, K.M., van Maris, A.J., Kotter, P., Luttik, M.A., van Dijken, J.P. & Pronk, J.T. (2001). Stoichiometry and compartmentation of nadh metabolism in saccharomyces cerevisiae. *FEMS Microbiol Rev*, **25**, 15–37.

Banuelos, M., Gancedo, C. & Gancedo, J.M. (1977). Activation by phosphate of yeast phosphofructokinase. *J Biol Chem*, **252**, 6394–8.

Barabasi, A.L. & Albert, R. (1999). Emergence of scaling in random networks. *Science*, **286**, 509–12.

Bartrons, R., Van Schaftingen, E., Vissers, S. & Hers, H.G. (1982). The stimulation of yeast phosphofructokinase by fructose 2,6-bisphosphate. *FEBS Lett*, **143**, 137–40.

Betz, A. & Chance, B. (1965). Phase relationship of glycolytic intermediates in yeast cells with oscillatory metabolic control. *Arch Biochem Biophys*, **109**, 585–94.

Bisson, L.F. & Fraenkel, D.G. (1983). Involvement of kinases in glucose and fructose uptake by saccharomyces cerevisiae. *Proc Natl Acad Sci U S A*, **80**, 1730–4.

Blazquez, M.A., Lagunas, R., Gancedo, C. & Gancedo, J.M. (1993). Trehalose-6-phosphate, a new regulator of yeast glycolysis that inhibits hexokinases. *FEBS Lett*, **329**, 51–4.

Boiteux, A. & Busse, H.G. (1989). Circuit analysis of the oscillatory state in glycolysis. *Biosystems*, **22**, 231–40.

Boiteux, A. & Hess, B. (1981). Design of glycolysis. *Philos Trans R Soc Lond B Biol Sci*, **293**, 5–22.

Boiteux, A., Goldbeter, A. & Hess, B. (1975). Control of oscillating glycolysis of yeast by stochastic, periodic, and steady source of substrate: a model and experimental study. *Proc Natl Acad Sci U S A*, **72**, 3829–33.

Boles, E. & Hollenberg, C.P. (1997). The molecular genetics of hexose transport in yeasts. *FEMS Microbiol Rev*, **21**, 85–111.

Boles, E., Heinisch, J. & Zimmermann, F.K. (1993). Different signals control the activation of glycolysis in the yeast saccharomyces cerevisiae. *Yeast*, **9**, 761–70.

Bonnet, J.A., de Kok, H.E. & Roels, J.A. (1980). The growth of saccharomyces cerevisiae cbs 426 on mixtures of glucose and ethanol: a model. *Antonie Van Leeuwenhoek*, **46**, 565–76.

Bro, C., Regenberg, B., Lagniel, G., Labarre, J., Montero-Lomel, M. & Nielsen, J. (2003). Transcriptional, proteomics, and metabolic responses to lithium in galactose-grown yeast cells. *Journal of Biological Chemistry*, **278**, 32141–32149.

Burrows, S. (1979). Baker's yeast. In A.H. Rose, ed., *Economic Microbiology*, 31–64, Academic Press, New York.

Castrillo, J.I. & Oliver, S.G. (2004). Yeast as a touchstone in post-genomic research: strategies for integrative analysis in functional genomics. *J Biochem Mol Biol*, **37**, 93–106.

Chambers, A., Packham, E.A. & Graham, I.R. (1995). Control of glycolytic gene expression in the budding yeast (saccharomyces cerevisiae). *Curr Genet*, **29**, 1–9.

Chance, B., Estabrook, R.W. & Ghosh, A. (1964a). Damped sinusoidal oscillations of cytoplasmic reduced pyridine nucleotide in yeast cells. *Proc Natl Acad Sci U S A*, **51**, 1244–51.

Chance, B., Ghosh, A., Higgins, J.J. & Maitra, P.K. (1964b). Cyclic and oscillatory responses of metabolic pathways involving chemical feedback and their computer representations. *Ann N Y Acad Sci*, **115**, 1010–24.

Christensen, B. & Nielsen, J. (2000). Metabolic network analysis. a powerful tool in metabolic engineering. *Adv Biochem Eng Biotechnol*, **66**, 209–31.

Clark, B.L. (1980). Stability of complex reaction networks. *Advances in Chemical Physics*, **43**, 1–215.

Clifton, D. & Fraenkel, D.G. (1981). The gcr (glycolysis regulation) mutation of saccharomyces cerevisiae. *J Biol Chem*, **256**, 13074–8.

Cornish-Bowden, A. (1995). *Fundamentals of Enzyme Kinetics*. Portland Press Ltd., London.

Daran-Lapujade, P., Jansen, M.L., Daran, J.M., van Gulik, W., de Winde, J.H. & Pronk, J.T. (2004). Role of transcriptional regulation in controlling fluxes in central carbon metabolism of saccharomyces cerevisiae. a chemostat culture study. *J Biol Chem*, **279**, 9125–38.

Davies, S.E. & Brindle, K.M. (1992). Effects of overexpression of phosphofructokinase on glycolysis in the yeast saccharomyces cerevisiae. *Biochemistry*, **31**, 4729–35.

de Koning, W. & van Dam, K. (1992). A method for the determination of changes of glycolytic metabolites in yeast on a subsecond time scale using extraction at neutral ph. *Anal Biochem*, **204**, 118–23.

de la Cruz, B.J., Prieto, S. & Scheffler, I.E. (2002). The role of the 5' untranslated region (utr) in glucose-dependent mrna decay. *Yeast*, **19**, 887–902.

Delgado, J., Meruane, J. & Liao, J.C. (1993). Experimental determination of flux control distribution in biochemical systems: In vitro model to analyze transient metabolite concentrations. *Biotechnology and Bioengineering*, **41**, 1121–1128.

deRisi, J., Iyer, V. & Brown, P. (1997). Exploring the metabolic and genetic control of gene expression on a genomic scale. *Science*, **278**, 680–686.

DeVit, M.J. & Johnston, M. (1999). The nuclear exportin msn5 is required for nuclear export of the mig1 glucose repressor of saccharomyces cerevisiae. *Curr Biol*, **9**, 1231–41.

Diderich, J.A., Schepper, M., van Hoek, P., Luttik, M.A., van Dijken, J.P., Pronk, J.T., Klaassen, P., Boelens, H.F., de Mattos, M.J., van Dam, K. & Kruckeberg, A.L. (1999a). Glucose uptake kinetics and transcription of hxt genes in chemostat cultures of saccharomyces cerevisiae. *J Biol Chem*, **274**, 15350–9.

Diderich, J.A., Teusink, B., Valkier, J., Anjos, J., Spencer-Martins, I., van Dam, K. & Walsh, M.C. (1999b). Strategies to determine the extent of control exerted by glucose transport on glycolytic flux in the yeast saccharomyces bayanus. *Microbiology*, **145 (Pt 12)**, 3447–54.

Diderich, J.A., Raamsdonk, L.M., Kruckeberg, A.L., Berden, J.A. & Van Dam, K. (2001). Physiological properties of saccharomyces cerevisiae from which hexokinase ii has been deleted. *Appl Environ Microbiol*, **67**, 1587–93.

Edwards, J.S. & Palsson, B.O. (1999). Systems properties of the haemophilus influenzae rd metabolic genotype. *J Biol Chem*, **274**, 17410–6.

Edwards, J.S. & Palsson, B.O. (2000). The escherichia coli mg1655 in silico metabolic genotype: its definition, characteristics, and capabilities. *Proc Natl Acad Sci U S A*, **97**, 5528–33.

Entian, K.D. & Zimmermann, F.K. (1980). Glycolytic enzymes and intermediates in carbon catabolite repression mutants of saccharomyces cerevisiae. *Mol Gen Genet*, **177**, 345–50.

Estruch, F., Treitel, M.A., Yang, X. & Carlson, M. (1992). N-terminal mutations modulate yeast snf1 protein kinase function. *Genetics*, **132**, 639–50.

Even, S., Lindley, N.D. & Cocaign-Bousquet, M. (2003). Transcriptional, translational and metabolic regulation of glycolysis in lactococcus lactis subsp. cremoris mg 1363 grown in continuous acidic cultures. *Microbiology*, **149**, 1935–44.

Famili, I., Forster, J., Nielsen, J. & Palsson, B.O. (2003). Saccharomyces cerevisiae phenotypes can be predicted by using constraint-based analysis of a genome-scale reconstructed metabolic network. *Proc Natl Acad Sci U S A*, **100**, 13134–9.

Fell, D.A. (1992). Metabolic control analysis: a survey of its theoretical and experimental development. *Biochem J*, **286**, 313–30.

Fell, D.A. (1997). *Understanding the Control of Metabolism*. Frontiers in Metabolism 2, Portland Press Ltd., London.

Fell, D.A. & Thomas, S. (1995). Physiological control of metabolic flux: the requirement for multisite modulation. *Biochem J*, **311**, 35–9.

Fell, D.A. & Wagner, A. (2000). The small world of metabolism. *Nat Biotechnol*, **18**, 1121–2.

Ficarro, S., McCleland, M., Stukenberg, P., Burke, D., Ross, M., Shabanowitz, J., Hunt, D. & White, F. (2002). Phosphoproteome analysis by mass spectrometry and its application to saccharomyces cerevisiae. *Nature Biotechnology*, **20**, 301–305.

Forster, J., Famili, I., Fu, P., Palsson, B. & Nielsen, J. (2003). Genome-scale reconstruction of the saccharomyces cerevisiae metabolic network. *Genome Research*, **13**, 244–253.

Fuhrmann, G.F., Volker, B., Sander, S. & Potthast, M. (1989). Kinetic analysis and simulation of glucose transport in plasma membrane vesicles of glucose-repressed and derepressed saccharomyces cerevisiae cells. *Experientia*, **45**, 1018–23.

Gaba, A., Wang, Z., Krishnamoorthy, T., Hinnebusch, A.G. & Sachs, M.S. (2001). Physical evidence for distinct mechanisms of translational control by upstream open reading frames. *Embo J*, **20**, 6453–63.

Galazzo, J.L. & Bailey, J.E. (1990). Fermentation pathway kinetics and metabolic flux control in suspended and immobilized saccharomyces cerevisiae. *Enzyme and Microbial Technology*, **12**, 162–172.

Gancedo, C. & Serrano, S. (1989). Energy-yielding metabolism. In A.H. Rose & J.S. Harrison, eds., *The Yeasts*, vol. 3, 205–259, Academic Press, New York.

Gancedo, J.M. (1998). Yeast carbon catabolite repression. *Microbiol Mol Biol Rev*, **62**, 334–61.

Ghosh, A. & Chance, B. (1964). Oscillations of glycolytic intermediates in yeast cells. *Biochem Biophys Res Commun*, **16**, 174–81.

Greenbaum, D., Colangelo, C., Williams, K. & Gerstein, M. (2003). Comparing protein abundance and mrna expression levels on a genomic scale. *Genome Biol*, **4**, 117.1–117.8.

Griffin, T., Gygi, S., Ideker, T., Rist, B., Eng, J., Hood, L. & Aebersold, R. (2002). Complementary profiling of gene expression at the transcriptome and proteome levels in saccharomyces cerevisiae. *Mol Cell Proteomics*, **1**, 323–333.

Groen, A.K., van Roermund, C.W., Vervoorn, R.C. & Tager, J.M. (1986). Control of gluconeogenesis in rat liver cells. flux control coefficients of the enzymes in the gluconeogenic pathway in the absence and presence of glucagon. *Biochem J*, **237**, 379–89.

Hauf, J., Zimmermann, F.K. & Muller, S. (2000). Simultaneous genomic overexpression of seven glycolytic enzymes in the yeast saccharomyces cerevisiae. *Enzyme Microb Technol*, **26**, 688–698.

Heinisch, J. (1986). Isolation and characterization of the two structural genes coding for phosphofructokinase in yeast. *Mol Gen Genet*, **202**, 75–82.

Heinrich, R. & Rapoport, T.A. (1973). Linear theory of enzymatic chains; its application for the analysis of the crossover theorem and of the glycolysis of human erythrocytes. *Acta Biol Med Ger*, **31**, 479–94.

Heinrich, R. & Rapoport, T.A. (1974). A linear steady-state treatment of enzymatic chains. general properties, control and effector strength. *Eur J Biochem*, **42**, 89–95.

Heinrich, R. & Schuster, S. (1996). *The Regulation of Cellular Systems*. Cahpman & Hall, New York.

Heinrich, R., Rapoport, S.M. & Rapoport, T.A. (1977). Metabolic regulation and mathematical models. *Prog Biophys Mol Biol*, **32**, 1–82.

Hess, B. & Boiteux, A. (1968). Mechanism of glycolytic oscillation in yeast. i. aerobic and anaerobic growth conditions for obtaining glycolytic oscillation. *Hoppe Seylers Z Physiol Chem*, **349**, 1567–74.

Hinnebusch, A. (2005). Translational regulation of gcn4 and the general amino acid control of yeast. *Annual Review of Microbiology*, **59**.

Hofmeyr, J.H. (1995). Metabolic regulation: a control analytic perspective. *J Bioenerg Biomembr*, **27**, 479–90.

Hofmeyr, J.H. & Cornish-Bowden, A. (1991). Quantitative assessment of regulation in metabolic systems. *Eur J Biochem*, **200**, 223–36.

Hohmann, S., Winderickx, J., de Winde, J., Valckx, D., Cobbaert, P., Luyten, K., de Meirsman, C., Ramos, J. & Thevelein, J. (1999). Novel alleles of yeast hexokinase pii with distinct effects on catalytic activity and catabolite repression of suc2. *Microbiology*, **145**, 703–714.

Holms, W.H. (1986). The central metabolic pathways of escherichia coli: relationship between flux and control at a branch point, efficiency of conversion to biomass, and excretion of acetate. *Curr Top Cell Regul*, **28**, 69–105.

Hubbard, J.H. & West, B.H. (1995). *Differential Equations: A Dynamical Systems Approach: Higher-Dimensional Systems.*, vol. 18 of *Texts in Applied Mathematics*. Springer-Verlag, New York.

Hynne, F., Dano, S. & Sorensen, P.G. (2001). Full-scale model of glycolysis in saccharomyces cerevisiae. *Biophys Chem*, **94**, 121–63.

Ideker, T., Thorsson, V., Ranish, J., Christmas, R., Buhler, J., Eng, J., Bumgarner, R., Goodlett, D., Aebersold, R. & Hood, L. (2001). Integrated genomic and proteomic analyses of a systematically perturbed metabolic network. *Science*, **292**, 929–934.

Jansen, M.L., Diderich, J.A., Mashego, M., Hassane, A., de Winde, J.H., Daran-Lapujade, P. & Pronk, J.T. (2005). Prolonged selection in aerobic, glucose-limited chemostat cultures of saccharomyces cerevisiae causes a partial loss of glycolytic capacity. *Microbiology*, **151**, 1657–1669.

Kacser, H. & Burns, J.A. (1973). The control of flux. *Symp Soc Exp Biol*, **27**, 65–104.

Kahn, D. & Westerhoff, H.V. (1991). Control theory of regulatory cascades. *J Theor Biol*, **153**, 255–85.

Kahn, D. & Westerhoff, H.V. (1993). The regulatory strength: how to be precise about regulation and homeostasis. *Acta Biotheor*, **41**, 85–96.

Koenig, S., Svergun, D., Koch, M.H.J., Huebner, G. & Schellenberger, A. (1992). Synchrotron radiation solution x-ray scattering study of the ph-dependence of the quarternary structure of yeast pyruvate decarboxylase. *Biochemistry*, **31**, 8726–8731.

Kolkman, A., Daran-Lapujade, P., Fullaondo, A., Olsthoorn, M., Pronk, J., Slijper, M. & Heck, A. (2006). Proteome analysis of yeast response to various nutrient limitations. **In press**.

Kotyk, A. (1967). Mobility of the free and of the loaded monosaccharide carrier in saccharomyces cerevisiae. *Biochim Biophys Acta*, **135**, 112–9.

Kraakman, L., Winderickx, J., Thevelein, J.M. & De Winde, J.H. (1999). Structure-function analysis of yeast hexokinase: structural requirements for triggering camp signalling and catabolite repression. *Biochem J*, **343**, 159–68.

Krampe, S. & Boles, E. (2002). Starvation-induced degradation of yeast hexose transporter hxt7p is dependent on endocytosis, autophagy and the terminal sequences of the permease. *FEBS Lett*, **513**, 193–6.

Kriegel, T.M., Rush, J., Vojtek, A.B., Clifton, D. & Fraenkel, D.G. (1994). In vivo phosphorylation site of hexokinase 2 in saccharomyces cerevisiae. *Biochemistry*, **33**, 148–52.

Kruckeberg, A.L. (1996). The hexose transporter family of saccharomyces cerevisiae. *Arch Microbiol*, **166**, 283–92.

Lagunas, R. (1993). Sugar transport in saccharomyces cerevisiae. *FEMS Microbiol Rev*, **10**, 229–42.

Lang, J.M. & Cirillo, V.P. (1987). Glucose transport in a kinaseless saccharomyces cerevisiae mutant. *J Bacteriol*, **169**, 2932–7.

Lange, H.C. & Heijnen, J.J. (2001). Statistical reconciliation of the elemental and molecular biomass composition of saccharomyces cerevisiae. *Biotechnol Bioeng*, **75**, 334–44.

Larsen, M.R., Larsen, P.M., Fey, S.J. & Roepstorff, P. (2001). Characterization of differently processed forms of enolase 2 from saccharomyces cerevisiae by two-dimensional gel electrophoresis and mass spectrometry. *Electrophoresis*, **22**, 566–75.

Lin, S.J., Kaeberlein, M., Andalis, A.A., Sturtz, L.A., Defossez, P.A., Culotta, V.C., Fink, G.R. & Guarente, L. (2002). Calorie restriction extends saccharomyces cerevisiae lifespan by increasing respiration. *Nature*, **418**, 344–8.

Ludin, K., Jiang, R. & Carlson, M. (1998). Glucose-regulated interaction of a regulatory subunit of protein phosphatase 1 with the snf1 protein kinase in saccharomyces cerevisiae. *Proc Natl Acad Sci U S A*, **95**, 6245–50.

Ma, H., Bloom, L.M., Walsh, C.T. & Botstein, D. (1989). The residual enzymatic phosphorylation activity of hexokinase ii mutants is correlated with glucose repression in saccharomyces cerevisiae. *Mol Cell Biol*, **9**, 5643–9.

Mashego, M.R., van Gulik, W.M., Vinke, J.L., Visser, D. & Heijnen, J.J. (2006). In vivo kinetics with rapid perturbation experiments in saccharomyces cerevisiae using a second-generation bioscope. *Metab Eng*, **8**, 370–83.

McCarthy, J.E. (1998). Posttranscriptional control of gene expression in yeast. *Microbiol Mol Biol Rev*, **62**, 1492–553.

Michels, C.A. & Romanowski, A. (1980). Pleiotropic glucose repression-resistant mutation in saccharomyces carlesbergensis. *J Bacteriol*, **143**, 674–9.

Monod, J., Changeux, J.P. & Jacob, F. (1963). Allosteric proteins and cellular control systems. *J Mol Biol*, **6**, 306–29.

Murcott, T.H., Gutfreund, H. & Muirhead, H. (1992). The cooperative binding of fructose-1,6-bisphosphate to yeast pyruvate kinase. *Embo J*, **11**, 3811–4.

Nilsson, A., Norbeck, J., Oelz, R., Blomberg, A. & Gustafsson, L. (2001a). Fermentative capacity after cold storage of baker's yeast is dependent on the initial physiological state but not correlated to the levels of glycolytic enzymes. *Int J Food Microbiol*, **71**, 111–24.

Nilsson, A., Pahlman, I.L., Jovall, P.A., Blomberg, A., Larsson, C. & Gustafsson, L. (2001b). The catabolic capacity of saccharomyces cerevisiae is preserved to a higher extent during carbon compared to nitrogen starvation. *Yeast*, **18**, 1371–81.

Ostling, J. & Ronne, H. (1998). Negative control of the mig1p repressor by snf1p-dependent phosphorylation in the absence of glucose. *Eur J Biochem*, **252**, 162–8.

Ozcan, S. & Johnston, M. (1999). Function and regulation of yeast hexose transporters. *Microbiol Mol Biol Rev*, **63**, 554–69.

Ozcan, S., Leong, T. & Johnston, M. (1996). Rgt1p of saccharomyces cerevisiae, a key regulator of glucose-induced genes, is both an activator and a repressor of transcription. *Mol Cell Biol*, **16**, 6419–26.

Ozcan, S., Dover, J. & Johnston, M. (1998). Glucose sensing and signaling by two glucose receptors in the yeast saccharomyces cerevisiae. *Embo J*, **17**, 2566–73.

Palsson, B.O. (2006). *Systems Biology: properties of reconstructed networks*. Cambridge University Press, Cambridge.

Parrou, J.L. & Francois, J. (1997). A simplified procedure for a rapid and reliable assay of both glycogen and trehalose in whole yeast cells. *Anal Biochem*, **248**, 186–8.

Petit, T., Diderich, J.A., Kruckeberg, A.L., Gancedo, C. & Van Dam, K. (2000). Hexokinase regulates kinetics of glucose transport and expression of genes encoding hexose transporters in saccharomyces cerevisiae. *J Bacteriol*, **182**, 6815–8.

Piper, M., Daran-Lapujade, P., Bro, C., Regenberg, B., Knudsen, S., Nielsen, J. & Pronk, J. (2002). Reproducibility of oligonucleotide microarray transcriptome analyses: an interlaboratory comparison using chemostat cultures of saccharomyces cerevsiae. *Journal of Biological Chemistry*, **277**, 37001–37008.

Portela, P., Howell, S., Moreno, S. & Rossi, S. (2002). In vivo and in vitro phosphorylation of two isoforms of yeast pyruvate kinase by protein kinase a. *J Biol Chem*, **277**, 30477–87.

Postma, E., Verduyn, C., Scheffers, W. & van Dijken, J. (1989). Enzymic analysis of the crabtree effect in glucose-limited chemostat cultures of saccharomyces cerevisiae. *Applied and Environmental Microbiology*, **55**, 468–477.

Pratt, J.M., Petty, J., Riba-Garcia, I., Robertson, D.H., Gaskell, S.J., Oliver, S.G. & Beynon, R.J. (2002). Dynamics of protein turnover, a missing dimension in proteomics. *Mol Cell Proteomics*, **1**, 579–91.

Pronk, J.T., Yde Steensma, H. & Van Dijken, J.P. (1996). Pyruvate metabolism in saccharomyces cerevisiae. *Yeast*, **12**, 1607–33.

Ptacek, J., Devgan, G., Michaud, G., Zhu, H., Zhu, X., Fasolo, J., Guo, H., Jona, G., Breitkreutz, A., Sopko, R., McCartney, R., Schmidt, M., Rachidi, N., Lee, S., Mah, A., Meng, L., Stark, M., Stern, D.F., de Virgilio, C., Tyers, M., Andrews, B., Gerstein, M., Schweitzer, B., Predki, P. & Snyder, M. (2005). Global analysis of protein phosphorylation in yeast. *Nature*, **438**, 679–684.

Raamsdonk, L.M. (2000). *Physiological Responses of Carbon Fluxes to Deletion of Specific Genes in Saccharomyces cerevisiae*. Ph.D. thesis, University of Amsterdam.

Raamsdonk, L.M., Diderich, J.A., Kuiper, A., van Gaalen, M., Kruckeberg, A.L., Berden, J.A. & Van Dam, K. (2001). Co-consumption of sugars or ethanol and glucose in a saccharomyces cerevisiae strain deleted in the hxk2 gene. *Yeast*, **18**, 1023–33.

Ramaiah, A. (1974). In B.L. Horecker & E. Stadtman, eds., *Current Topics in Cell Regulation*, vol. 8, 297–345, Academic Press, New York.

Randez-Gil, F., Herrero, P., Sanz, P., Prieto, J.A. & Moreno, F. (1998). Hexokinase pii has a double cytosolic-nuclear localisation in saccharomyces cerevisiae. *FEBS Lett*, **425**, 475–8.

Rayner, T., Gray, J. & Thorner, J. (2002). Direct and novel regulation of camp-dependent protein kinase by mck1p, a yeast glycogen synthase kinase-3. *Journal of Biological Chemistry*, **277**, 16814–16822.

Reder, C. (1988). Metabolic control theory: a structural approach. *J Theor Biol*, **135**, 175–201.

Reich, J.G. & Sel'kov, E.E. (1981). *Energy Metabolism of the Cell*. Academic Press, New York.

Reifenberger, E., Freidel, K. & Ciriacy, M. (1995). Identification of novel hxt genes in saccharomyces cerevisiae reveals the impact of individual hexose transporters on glycolytic flux. *Mol Microbiol*, **16**, 157–67.

Reifenberger, E., Boles, E. & Ciriacy, M. (1997). Kinetic characterization of individual hexose transporters of saccharomyces cerevisiae and their relation to the triggering mechanisms of glucose repression. *Eur J Biochem*, **245**, 324–33.

Reijenga, K.A., van Megen, Y.M., Kooi, B.W., Bakker, B.M., Snoep, J.L., van Verseveld, H.W. & Westerhoff, H.V. (2005). Yeast glycolytic oscillations that are not controlled by a single oscillophore: a new definition of oscillophore strength. *J Theor Biol*, **232**, 385–98.

Richter, O., Betz, A. & Giersch, C. (1975). The response of oscillating glycolysis to perturbations in the nadh/nad system: a comparison between experiments and a computer model. *Biosystems*, **7**, 137–46.

Rizzi, M., Baltes, M., Theobald, U. & Reuss, M. (1997). In vivo analysis of metabolic dynamics in saccharomyces cerevisiae: Ii. mathematical model. *Biotechnology and Bioengineering*, **55**, 598–608.

Rolland, F., Winderickx, J. & Thevelein, J.M. (2002). Glucose-sensing and -signalling mechanisms in yeast. *FEMS Yeast Res*, **2**, 183–201.

Rose, M., Albig, W. & Entian, K.D. (1991). Glucose repression in saccharomyces cerevisiae is directly associated with hexose phosphorylation by hexokinases pi and pii. *Eur J Biochem*, **199**, 511–8.

Rossell, S., van der Weijden, C.C., Kruckeberg, A., Bakker, B.M. & Westerhoff, H.V. (2002). Loss of fermentative capacity in baker's yeast can partly be explained by reduced glucose uptake capacity. *Mol Biol Rep*, **29**, 255–7.

Sanz, P., Alms, G.R., Haystead, T.A. & Carlson, M. (2000a). Regulatory interactions between the reg1-glc7 protein phosphatase and the snf1 protein kinase. *Mol Cell Biol*, **20**, 1321–8.

Sanz, P., Ludin, K. & Carlson, M. (2000b). Sip5 interacts with both the reg1/glc7 protein phosphatase and the snf1 protein kinase of saccharomyces cerevisiae. *Genetics*, **154**, 99–107.

Sato, T., Lopez, M.C., Sugioka, S., Jigami, Y., Baker, H.V. & Uemura, H. (1999). The e-box dna binding protein sgc1p suppresses the gcr2 mutation, which is involved in transcriptional activation of glycolytic genes in saccharomyces cerevisiae. *FEBS Lett*, **463**, 307–11.

Sauro, H.M. (1989). Regulatory responses and control analysis: Assesment of the relative importance of internal effectors. In A. Cornish-Bowden & M.L. Cardenas, eds., *Control of Metabolic Processes*, vol. 190 of *A*, 454, Plenum Press, New York.

Savageau, M.A. (1969a). Biochemical systems analysis. i. some mathematical properties of the rate law for the component enzymatic reactions. *J Theor Biol*, **25**, 365–9.

Savageau, M.A. (1969b). Biochemical systems analysis. ii. the steady-state solutions for an n-pool system using a power-law approximation. *J Theor Biol*, **25**, 370–9.

Savinell, J.M. & Palsson, B.O. (1992a). Network analysis of intermediary metabolism using linear optimization. i. development of mathematical formalism. *J Theor Biol*, **154**, 421–54.

Savinell, J.M. & Palsson, B.O. (1992b). Network analysis of intermediary metabolism using linear optimization. ii. interpretation of hybridoma cell metabolism. *J Theor Biol*, **154**, 455–73.

Schaaff, I., Heinisch, J. & Zimmermann, F.K. (1989). Overproduction of glycolytic enzymes in yeast. *Yeast*, **5**, 285–90.

Schilling, C.H., Letscher, D. & Palsson, B.O. (2000). Theory for the systemic definition of metabolic pathways and their use in interpreting metabolic function from a pathway-oriented perspective. *J Theor Biol*, **203**, 229–48.

Schilling, C.H., Covert, M.W., Famili, I., Church, G.M., Edwards, J.S. & Palsson, B.O. (2002). Genome-scale metabolic model of helicobacter pylori 26695. *J Bacteriol*, **184**, 4582–93.

Schlosser, P.M., Riedy, B. & Bailey, J.E. (1994). Ethanol production in bakers' yeast: Application of experimental perturbation techniques for model development and resultant changes in flux control analysis. *Biotechnology Progress*, **10**, 141–154.

Schuster, S., Fell, D.A. & Dandekar, T. (2000). A general definition of metabolic pathways useful for systematic organization and analysis of complex metabolic networks. *Nat Biotechnol*, **18**, 326–32.

Segel, I.H. (1993). *Enzyme Kinetics: Behavior and Analysis of Rapid Equilibrium and Steady-State Enzyme Systems*. John Wiley & Sons, Inc.

Shenton, D., Smirnova, J.B., Selley, J.N., Carroll, K., Hubbard, S.J., Pavitt, G.D., Ashe, M.P. & Grant, C.M. (2006). Global Translational Responses to Oxidative Stress Impact upon Multiple Levels of Protein Synthesis. *J Biol Chem*, **281**, 29011–29021.

Sherman, F. (1997). Yeast genetics. In R.A. Meyers, ed., *The Encyclopedia of Molecular Biology and Molecular Medicine*, vol. 6, 302–325, VCH Pub., Weinheim.

Shulman, R.G. & den Hollander, J. (2004). Futile cycling in yeast: How to control gluttony in the midst of plenty. In R.G. Shulman & D.L. Rothman, eds., *Metabolomics by in vivo NMR*, 206, John Wiley & Sons, Inc.

Shulman, R.G., Bloch, G. & Rothman, D.L. (1995). In vivo regulation of muscle glycogen synthase and the control of glycogen synthesis. *Proc Natl Acad Sci U S A*, **92**, 8535–42.

Skowyra, D., Craig, K.L., Tyers, M., Elledge, S.J. & Harper, J.W. (1997). F-box proteins are receptors that recruit phosphorylated substrates to the scf ubiquitin-ligase complex. *Cell*, **91**, 209–19.

Smirnova, J.B., Selley, J.N., Sanchez-Cabo, F., Carroll, K., Eddy, A.A., McCarthy, J.E., Hubbard, S.J., Pavitt, G.D., Grant, C.M. & Ashe, M.P. (2005). Global gene expression profiling reveals widespread yet distinctive translational responses to different eukaryotic translation initiation factor 2b-targeting stress pathways. *Mol Cell Biol*, **25**, 9340–9.

Smits, H., Hauf, J., Muller, S., Hobley, T., Zimmermann, F., Hahn-Hagerdal, B., Nielsen, J. & Olsson, L. (2000). Simultaneous overexpression of enzymes of the lower part of glycolysis can enhance the fermentative capacity of saccharomyces cerevisiae. *Yeast*, **16**, 1325–1334.

Smits, H.P., Smits, G.J., Postma, P.W., Walsh, M.C. & van Dam, K. (1996). High-affinity glucose uptake in saccharomyces cerevisiae is not dependent on the presence of glucose-phosphorylating enzymes. *Yeast*, **12**, 439–47.

Sols, A. (1981). Multimodulation of enzyme activity. *Curr Top Cell Regul*, **19**, 77–101.

Sols, A. & Salas, M.L. (1966). Phosphofructokinase: yeast. In W. Wood, ed., *Methods in Enzymology*, Academic Press, New York.

Stein, W. (1986). *Transport and Diffussion Across Membranes*. Academic Press, London.

Stephanopoulos, G. (1999). Metabolic fluxes and metabolic engineering. *Metab Eng*, **1**, 1–11.

Stouthamer, A.H. (1979). The search for correlation between theoretical and experimental grwoth yields. *Microbial Biochemistry*, **21**, 1–48.

Strang, G. (1986). *Linear Algebra and its Applications*. Harcourt Brace Jovanovich, San Diego, 3rd edn.

ter Kuile, B.H. (1996). Metabolic adaptation of trichomonas vaginalis to growth rate and glucose availability. *Microbiology*, **142**, 3337–45.

ter Kuile, B.H. & Westerhoff, H.V. (2001). Transcriptome meets metabolome: hierarchical and metabolic regulation of the glycolytic pathway. *FEBS Lett*, **500**, 169–71.

Teusink, B., Diderich, J.A., Westerhoff, H.V., van Dam, K. & Walsh, M.C. (1998). Intracellular glucose concentration in derepressed yeast cells consuming glucose is high enough to reduce the glucose transport rate by 50%. *J Bacteriol*, **180**, 556–62.

Teusink, B., Passarge, J., Reijenga, C.A., Esgalhado, E., van der Weijden, C.C., Schepper, M., Walsh, M.C., Bakker, B.M., van Dam, K., Westerhoff, H.V. & Snoep, J.L. (2000). Can yeast glycolysis be understood in terms of in vitro kinetics of the constituent enzymes? testing biochemistry. *Eur J Biochem*, **267**, 5313–29.

Thomsson, E., Larsson, C., Albers, E., Nilsson, A., Franzen, C.J. & Gustafsson, L. (2003). Carbon starvation can induce energy deprivation and loss of fermentative capacity in saccharomyces cerevisiae. *Appl Environ Microbiol*, **69**, 3251–7.

Treitel, M.A., Kuchin, S. & Carlson, M. (1998). Snf1 protein kinase regulates phosphorylation of the mig1 repressor in saccharomyces cerevisiae. *Mol Cell Biol*, **18**, 6273–80.

Tucker, M. & Parker, R. (2000). Mechanisms and control of mrna decapping in saccharomyces cerevisiae. *Annu Rev Biochem*, **69**, 571–95.

Uemura, H. & Fraenkel, D.G. (1990). gcr2, a new mutation affecting glycolytic gene expression in saccharomyces cerevisiae. *Mol Cell Biol*, **10**, 6389–96.

Uemura, H., Koshio, M., Inoue, Y., Lopez, M.C. & Baker, H.V. (1997). The role of gcr1p in the transcriptional activation of glycolytic genes in yeast saccharomyces cerevisiae. *Genetics*, **147**, 521–32.

van Gulik, W. & Heijnen, J.J. (1995). A metabolic network stoichioetry analysis of mocrobial growth and product formation. *Biotechnol Bioeng*, **48**, 681–696.

van Hoek, P. (2000). *Fermentative Capacity in Aerobic Cultures of Bakkers' yeast*. Ph.D. thesis, Delft University of Technology.

van Hoek, P., Flikweert, M.T., van der Aart, Q.J., Steensma, H.Y., van Dijken, J.P. & Pronk, J.T. (1998a). Effects of pyruvate decarboxylase overproduction on flux distribution at the pyruvate branch point in saccharomyces cerevisiae. *Appl Environ Microbiol*, **64**, 2133–40.

van Hoek, P., Van Dijken, J.P. & Pronk, J.T. (1998b). Effect of specific growth rate on fermentative capacity of baker's yeast. *Appl Environ Microbiol*, **64**, 4226–33.

van Hoek, P., van Dijken, J.P. & Pronk, J.T. (2000). Regulation of fermentative capacity and levels of glycolytic enzymes in chemostat cultures of saccharomyces cerevisiae. *Enzyme Microb Technol*, **26**, 724–736.

Vanrolleghem, P.A., de Jong-Gubbels, P., van Gulik, W.M., Pronk, J.T., van Dijken, J.P. & Heijnen, S. (1996). Validation of a metabolic network for saccharomyces cerevisiae using mixed substrate studies. *Biotechnol Prog*, **12**, 434–48.

Varma, A. & Palsson, B.O. (1994). Stoichiometric flux balance models quantitatively predict growth and metabolic by-product secretion in wild-type escherichia coli w3110. *Appl Environ Microbiol*, **60**, 3724–31.

Verduyn, C., Postma, E., Scheffers, W.A. & van Dijken, J.P. (1990a). Energetics of saccharomyces cerevisiae in anaerobic glucose-limited chemostat cultures. *J Gen Microbiol*, **136**, 405–12.

Verduyn, C., Postma, E., Scheffers, W.A. & van Dijken, J.P. (1990b). Physiology of saccharomyces cerevisiae in anaerobic glucose-limited chemostat cultures. *J Gen Microbiol*, **136**, 395–403.

Verduyn, C., Stouthamer, A.H., Scheffers, W.A. & van Dijken, J.P. (1991). A theoretical evaluation of growth yields of yeasts. *Antonie Van Leeuwenhoek*, **59**, 49–63.

Verduyn, C., Postma, E., Scheffers, W.A. & Van Dijken, J.P. (1992). Effect of benzoic acid on metabolic fluxes in yeasts: a continuous-culture study on the regulation of respiration and alcoholic fermentation. *Yeast*, **8**, 501–17.

Vilela, C. & McCarthy, J.E. (2003). Regulation of fungal gene expression via short open reading frames in the mrna 5'untranslated region. *Mol Microbiol*, **49**, 859–67.

Vilela, C., Linz, B., Rodrigues-Pousada, C. & McCarthy, J. (1998). The yeast transcription factor genes yap1 and yap2 are subject to differential control at the levels of both translation and mrna stability. *Nucl Acids Res*, **26**, 1150–1159.

Vojtek, A.B. & Fraenkel, D.G. (1990). Phosphorylation of yeast hexokinases. *Eur J Biochem*, **190**, 371–5.

Wagner, A. & Fell, D.A. (2001). The small world inside large metabolic networks. *Proc Biol Sci*, **268**, 1803–10.

Walsh, M.C., Smits, H.P., Scholte, M. & van Dam, K. (1994). Affinity of glucose transport in saccharomyces cerevisiae is modulated during growth on glucose. *J Bacteriol*, **176**, 953–8.

Washburn, M., Wolters, D. & Yates III, J. (2001). Large-scale analysis of the yeast proteome by multidimensional protein identification technology. *Nat Biotechnol*, **19**, 242–247.

Watts, D.J. & Strogatz, S.H. (1998). Collective dynamics of 'small-world' networks. *Nature*, **393**, 440–2.

Westerhoff, H.V. & Chen, Y.D. (1984). How do enzyme activities control metabolite concentrations? an additional theorem in the theory of metabolic control. *Eur J Biochem*, **142**, 425–30.

Westerhoff, H.V. & Van Dam, K. (1987). *Thermodynamics and control of biological free-energy transduction*. Elsevier Science Publishing Company, Inc., Amsterdam.

Westerhoff, H.V., Reijenga, K.A., Snoep, J.L., Kholodenko, B.N. & ter Kuile, B.H. (2000). A new millenium: functional genomics, cellular bioinformatics, regulation analysis. In J. Hofmeyr, J. Rohwer & J. Snoep, eds., *Animating the Cellular Map*, 1–7, Stellenbosch University Press, Stellenbosch.

Wieczorke, R., Krampe, S., Weierstall, T., Freidel, K., Hollenberg, C.P. & Boles, E. (1999). Concurrent knock-out of at least 20 transporter genes is required to block uptake of hexoses in saccharomyces cerevisiae. *FEBS Lett*, **464**, 123–8.

Wilson, W.A., Hawley, S.A. & Hardie, D.G. (1996). Glucose repression/derepression in budding yeast: Snf1 protein kinase is activated by phosphorylation under derepressing conditions, and this correlates with a high amp:atp ratio. *Curr Biol*, **6**, 1426–34.

Wilusz, C.J. & Wilusz, J. (2004). Bringing the role of mrna decay in the control of gene expression into focus. *Trends Genet*, **20**, 491–7.

Woods, A., Munday, M.R., Scott, J., Yang, X., Carlson, M. & Carling, D. (1994). Yeast snf1 is functionally related to mammalian amp-activated protein kinase and regulates acetyl-coa carboxylase in vivo. *J Biol Chem*, **269**, 19509–15.

Yang, C., Hua, Q. & Shimizu, K. (2002). Integration of the information from gene expression and metabolic fluxes for the analysis of the regulatory mechanisms in synechocystis. *Applied Microbiology and Biotechnology*, **58**, 813–822.

Zimmermann, F.K. & Scheel, I. (1977). Mutants of saccharomyces cerevisiae resistant to carbon catabolite repression. *Mol Gen Genet*, **154**, 75–82.

Samenvatting

Dit proefschrift gaat over de regulatieprocessen die en rol spelen in de aanpassing van metabole systemen aan hun omgeving en aan genetische veranderingen. Onderzoek aan regulatiemechanismen is uniek voor de biologie. Het richt zich op systemen waarvan de complexiteit ongeëvenaard is in niet-biologische systemen. Biologische systemen kunnen zich aanpassen: levende cellen moduleren de eigenschappen van hun systemen als reactie op veranderingen in hun omgeving. Deze aanpassingen zijn onderworpen aan tot dusverre onbekende drijevende krachten en beperkingen.

In het inleidende hoofdstuk (Hoofdstuk 1) wordt betoogd dat ons begrip van regulatieprocessen wordt beperkt door het ontbreken van een precieze definitie van het begrip regulatie en door het ontbreken van geschikte methodes om regulatie-processen op een eenduidige en kwantitatieve manier te beschijven. De hoofdstukken 2 en 5 bescrijven de implementatie, evaluatie en verdere ontwikkeling van Regulatie Analyse, een methode die een kwantitatieve beschrijving van de regulatie van enzymsnelheden en hun katalytische capaciteiten mogelijk maakt. Deze methode is geïmplementeerd, getest en uitgewerkt in een serie van onderzoeken naar de regulatie van de glycolyse in *Saccharomyces cerevisiae* tijdens nutriënt-starvatie, zuurstof-deprivatie, toegenomen dissipatie van vrije energie door toevoeging van benzoëzuur, of na deletie van het *HXK2* gen dat codeert voor hexokinase II. De experimentele bevindingen en analyses, die in dit proefschrift beschreven worden, brengen nieuwe inzichten in de complexiteit van de regulatie van de metabole flux en de katalytische capaciteiten van de enzymen die de reacties katalyseren.

In het verleden zijn er verscheidene pogingen gedaan om een kwantitatief kader te ontwerpen voor de studie naar metabole regulatie. Daaruit is Regulatie Analyse naar voren gekomen als de methode die het meest passend is voor *experimenteel* onderzoek naar regulatieprocessen. Regulatie Analyse ontleedt kwantitatief de bijdragen van veranderingen in enzym-capaciteiten (V_{max} -hiërarchische regulatie genoemd) en veranderingen in de interactie van enzymen met de rest van het metabolisme (metabole regulatie genoemd) op de lokale regulatie van de enzymsnelheden. Deze verdeling is gebaseerd op een eigenschap van de meeste enzymgekatalyseerde reacties: de snelheid van de katalyse is recht evenredig met de hoeveelheid van het actieve enzym. Regulatie Analyse maakt het mogelijk om eenduidige en kwantitatieve beschrijvingen te geven van de regulatie van de flux door de betrokken enzymen, onafhankelijk van de complexiteit van het systeem of het type verstoring.

Regulatie Analyse wordt door dit gehele proefschrift gebruikt om de regulatie van de flux door de individuele enzymen betrokken bij de glycolyse en fermentatie in de

gist *Saccharmoyces cerevisiae* te beschrijven wanneer de gist zich aanpast aan verscheidene veranderingen van de omgeving en van zijn eigen genetische samenstelling. Hoofdstuk 2 geeft een gedetailleerde beschrijving van de methode en introduceert precieze biochemische interpretaties voor alle mogelijke numerieke uitkomsten van de analyse. Verder verfijnt het de oorspronkelijke interpretatie van de hiërarchische en metabole regulatie zodanig dat ook de differentiële expressie van iso-enzymen met verschillende kinetische eigenschappen is ondergebracht.

In Hoofdstuk 3 is Regulatie Analyse gebruikt om drie toetsbare hypotheses over de globale regulatie van de flux door de gehele route te formuleren in termen van de lokale regulatie van de enzymsnelheden in die route. De eerste hypothese voorspelt dat alle enzymsnelheden metabool worden gereguleerd (d.w.z. volledige afwezigheid van hiërarchische regulatie), De tweede hypothese voorspelt dat een enkel 'sleutelenzym' in de route hiërarchisch wordt gereguleerd terwijl alle andere stappen metabool worden gereguleerd. De derde hypothese voorspelt uitsluitend hiërarchische regulatie van alle enzymsnelheden in de route om de homeostase van de metaboliet-concentraties te garanderen. De hoofdstukken 3, 4 en 5 beschrijven de regulatie van de flux door de individuele enzymen betrokken bij de glycolyse en fermentatie wanneer *Saccharomyces cerevisiae* zich aanpast aan: stikstof- of koolstof-starvatie, zuurstof-deprivatie, toegenomen dissipatie van vrije energie door toevoeging van benzoëzuur en tenslotte aan de deletie van het gen *HXK2* dat codeert voor hexokinase II. Gezamenlijk leiden deze studies tot de volgende conclusies: (i) metabole regulatie levert vaak een belangrijke bijdrage aan de lokale regulatie van de enzymsnelheden, (ii) levende gistcellen gebruiken alle mogelijke combinaties van hiërarchische en metabole regulatie om de snelheden van de individuele enzymen te moduleren, (iii) de flux door enzymen in dezelfde route wordt vaak op verschillende manieren gereguleerd, wat suggereert dat de enzymen verschillende rollen spelen in de regulatie van de flux door de gehele route, (iv) dezelfde metabole stap is vaak verschillend gereguleerd wanneer de cellen zich aanpassen aan verschillende verstoringen, (v) alle voorgestelde hypotheses over de globale regulatie van de flux door de gehele route zijn weerlegd voor de onderzocht kweekomstandigheden, wat impliceert dat ze niet algemeen geldig zijn, en (vi) de regulatie van de glycolytische en fermentatieve flux is vaak gereguleerd door veranderingen van enzyme binnen èn buiten de route. Deze bevindingen suggereren dat de flux door de gehele route op een subtiele manier wordt gereguleerd, -waarbij de verschillende enzymen een verschillende regulerende rol spelen. Ze laten zien dat de regulatie van de flux door de gehele route niet noodzakerwijs wordt bepaald door een enkele drijvende kracht of beperking. Ze maken het ook noodzakelijk on nieuwe hypotheses te formuleren over de globale regulatie van de flux door de gehele route.

Een uitbreiding van de Regulatie Analyse wordt beschreven in Hoofdstuk 4. Hiermee kan de regulatie van enzymhoeveelheden en katalytische capaciteiten worden beschreven in termen van de bijdragen van veranderingen in mRNA concentraties, translatie- en eiwit-degradatie snelheden en posttranslationele modificaties. De analyse is gebaseerd op de aanname dat eiwitconcentraties in steady-state zijn en dat de snelheden van translatie en degradatie van individuele eiwitten recht evenredig zijn met de corresponderende concentraties van mRNA en eiwit. De eerste aanname is waarschijnlijk gegarandeerd door het gebruik van chemostaat-cultures, maar de andere dient nog experimenteel geverifieerd te woorden. Hiertoe is een grotere

nauwkeurigheid van beschikbare analytische technieken vereist. De aanname dat translatie- en eiwit-degradatie-snelheden recht evenredig zijn met de concentraties van respectievelijk het corresponderend mRNA en het eiwit, zijn gebaseerd op de verwachting dat de betrokken enzymcomplexen (ribosomen en proteasoom) niet specifiek zijn en dat de concentratie van een enkel mRNA of eiwit een minderheid vertegenwoordigt in de populaties van alle mRNAs of eiwitten.

In Hoofdstuk 4 wordt deze uitgebreide Regulatie Analyse toegepast op de regulatie van de hoeveelheden en capaciteiten van glycolytisch enzymen, wanneer *S. cerevisiae* zich aanpast aan anarobiosis of de aanwezigheid van benzoëzuur. De experimenten laten zien dat veranderingen in mRNA concentraties slecht overeenkomen met de veranderingen in hoeveelheden en capaciteiten van de enzymen. De analyse suggereert dat veranderingen in mRNA voor minder dan 50% bijdragen aan de regulatie van de hoeveelheden en capaciteiten van de glycolytische enzymen en dat veranderingen in de snelheden van translatie en/of eiwit-degradatie de belangrijkste regulatoren van de hoeveelheden eiwit zijn. Pogingen om de bijdrage van posttranslationele modificaties aan de regulatie van enzym-capaciteiten te kwantificeren, tonen aan dat een meer nauwkeurige en reproduceerbare proteoom-analyse noodzakelijk is. Hoewel de experimentele onzekerheid te groot was om definitieve om conclusies kunnen te trekken, geven de data de indruk dat er posttranslationele modificaties plaatsvinden die de katalytische capaciteit van triosefosfaat-isomerase en fosfoglyceraat-kinase beïnvloeden.

De methoden die zijn ontwikkeld en gebruikt in dit proefschrift, leveren een kwantitatief kader op waarmee hypotheses over regulatieprocessen experimenteel kunnen worden getoest. De toepassing van deze methoden om de werkelijke regulatieprocessen in levende cellen te beschrijven heeft ons inzicht gegeven in een eerder onbeschreven complexiteit van de processen die het metabolisme reguleren. Deze studie laat zien dat processen waaraan relatief weinig aandacht is besteed, zoals metabole regulatie en translatie- en eiwit-degradatie, waarschijnlijk een belangrijke rol spelen in de regulatie van metabole systemen.

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