Exposing a complex metabolic system: glycolysis in Saccharomyces cerevisiae
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CHAPTER 1

GENERAL INTRODUCTION

The ultimate objective of the life sciences is the understanding of the behavior of organisms. Part of this objective is to understand this behavior on the basis of their molecular components and the interactions thereof. Component identification is one of the first steps towards such a goal. Today all genetic components that constitute the unicellular eucaryote Saccharomyces cerevisiae (Baker's yeast) have been identified thanks to a joint effort to sequence its complete genome [1, 242, 264]. Baker's yeast contains about 6000 genes [121], encoding proteins that are required for DNA replication, transcription and translation, for signal transduction, for free-energy transduction and for biosynthesis. Approximately half of the 6000 genes of yeast, however, are 'ORFans' [88], i.e., have a sequence that has no homology to any other gene for which the function is known. Hence, their sequence reveals very little or nothing at all about their biological function. Accordingly, some 3000 yeast proteins have been identified in terms of their existence, but not in terms of their function. With identification of function we here mean the activity carried out by the protein, e.g. transport of amino acids, or protein kinase activity involved in the regulatory cascade in response to oxidative stress.

A vast number of researchers is looking for and characterizing genes responsible for a particular phenotype. In fact, one might compare today's hunt for gene function with the hunt in the first half of this century for the function of metabolic intermediates and enzymes. This hunt resulted in the biochemical pathways as we now know them. In essence either hunt is aimed at identification of the function of components. For example, the EUROFAN project aims at the elucidation of the function of some 1,000 novel components [264, 265]. This is an immense challenge, and the identification of many new components in yeast is bound to boost our understanding of living cells in general.

However, it is unthinkable that we will completely understand how a living cell functions - which is our ultimate objective - even when we have identified the (individual) function of all the components. Jensen et al. have compared the living cell with a television set [173]: even if one were to have all the basic components, like resistors, transistors and capacitors, and if one also were to have a circuit diagram (i.e. the 'stoichiometry'), one would still miss the WorldCup final when the magnitudes of the resistances and capacitances were not indicated on the diagram. "The proper voltages must arise at all positions and must respond correctly to changes in input
signals so as to produce the response that is optimal in functional terms”[173]. Even when all the essential components have been identified, true understanding of the function of a component within a system requires quantitative knowledge of how much of the component is present, how responsive the component is to changes in its immediate environment and how it interacts with the other components.

This thesis is concerned with this challenge. It addresses a limited part of a living organism, i.e. the regulation and control properties of metabolic pathways at the metabolic level. The model system under study is glycolysis in the yeast S. cerevisiae. There are good reasons to study this particular system. First, because all components are largely known and interactions between the components have been studied, we are in a position to see if we can get this television set to work (cf. above). Second, glycolysis is central to the metabolism of carbohydrates: it is an important source of free energy and delivers many precursors for biosynthesis of carbohydrates, lipids, amino acids and nucleotides. Third, yeast glycolysis is one of the best-defined biochemical systems that was shown to exhibit periodic behavior in the form of oscillations in the concentration of its intermediates. It is therefore an ideal system to extend the study of metabolism from the classical steady state behavior to the domain of dynamics. Fourth, it goes without saying that yeast glycolysis is an industrially important process and that a better understanding of its control and regulation will find its applications in both classical and modern biotechnology.

The most important reason to study yeast glycolysis is that its components and their interactions have been much studied over more than a century. So much even, that textbooks suggest that the questions about the control and regulation of glycolysis have all been solved long ago [350]. The answers to many of those questions, however, are based on qualitative principles that cannot stand the test of close (quantitative) inspection (as amply pointed out in Fell’s book on the control of metabolism [98]). One of these is the concept of a rate-limiting step (or pacemaker enzyme) which could be identified just on the basis of the properties of individual enzymes. Accordingly, the complex allosteric regulation of phosphofructokinase, combined with the thermodynamic irreversibility of the reaction it catalyzes and the position of the enzyme in the glycolytic chain (i.e. at the first committing step), made this enzyme a classical example of a pacemaker enzyme. Overexpression of this enzyme in yeast, however, did not result in an increase in the glycolytic rate [81, 319] (see below).

Since the development of Metabolic Control Analysis (MCA), a mathematical framework describing control and regulation in precise, quantitative terms, it is clear that control and regulation are systemic properties. They depend on the pathway architecture and the properties of all enzymes. In most systems studied so far, no single rate-limiting step can be found, but rather, all enzymes control the metabolic flux to some extent. MCA enables to understand the control properties of enzymes quantitatively.
Moreover, MCA offers clear definitions of control and regulation, concepts that have been polluted so much by imprecise use that the difference between them has lost meaning. In this thesis the concept of control is reserved for the potential of an enzyme to affect a system variable (such as a flux or the concentration of a metabolite) [98, 160]. Regulation is on a higher level of description [160]. It deals with matters such as responsiveness to external conditions, homeostasis and communication between enzymes in the metabolic network [182]. Discrimination between well-regulated and poorly-regulated metabolic systems requires a definition of performance and therefore requires specific assignment of function to the pathway. For example, if glycolysis is considered to function as a producer of ATP, it may be considered well regulated if it were to increase its flux when the concentration of ATP decreases.

The precise definitions provided by MCA have been used in this thesis to try to understand certain aspects of the regulation and control of yeast glycolysis quantitatively, both in steady state and during glycolytic oscillations. In this light I shall continue this General Introduction in the following way. First, in section 1.1 the main concepts of MCA are introduced to readers unfamiliar with the subject. Then in section 1.2 the occurrence of oscillations in metabolic systems is explained by stability analysis of steady states. In section 1.3 our current understanding and ignorance of yeast glycolysis is described. This section highlights important questions that are still unanswered. In section 1.4 the tools that are available to study metabolism quantitatively are described and their limitations discussed. Finally, in section 1.5, the contents of this thesis are outlined.

1.1 Metabolic Control Analysis

In this section, the basic concepts and theorems of Metabolic Control Analysis (MCA) will be described. An alternative (and partly overlapping) mathematical framework that deals with quantitative descriptions of metabolic pathways is offered by Biological Systems Theory [318]. Through the years MCA has become more widely used in the quantitative analysis of metabolic pathways. Biological Systems Theory has not been used in this thesis and I will therefore only discuss MCA and its applications. The introduction to MCA presented here is included to enable readers unfamiliar with this area to read this thesis without consulting additional literature. They are encouraged, however, to read Henrik Kacser's update of the original paper on MCA [180]) which is an exceptionally clear account that introduces the main concepts and terminology of the theory. A more complete overview of MCA is also given in some reviews [75, 97, 184, 331], and in two recent books on the control of metabolism [98, 146]. There are also

1 Sections 1.1.1 and 1.1.2 were published in modified form in collaboration with Frank Baganz, Hans V. Westerhoff and Steve G. Oliver, as “Metabolic Control Analysis as a tool in the elucidation of the function of novel genes” in: Tuite, M. F. and Brown, A. J. P. (Eds), Methods in Microbiol., Academic Press, pp. 297-336 (1998).
useful Internet sites dealing with MCA, including tutorials [P. Mendes: http://gepasi.dbs.aber.ac.uk/metab/mca_home.htm], a course [P. Butler: http://www.bi.umist.ac.uk/courses/2IRM/MCA/default.htm], a FAQ site [A. Cornish-Bowden: http://ir2lcb.cnrs-mrs.fr/lcbpage/athel/mcafaq.html], and a newsgroup [see, e.g., http://www.bio.net:80/hypermail/BTK-MCA/].

1.1.1 Definitions and concepts of Metabolic Control Analysis

To analyze the behavior of any system, it is important to distinguish between parameters and variables. Parameters are set, either by nature (e.g. kinetic constants) or by the experimenter (e.g. pH and temperature, or the concentration of some inhibitor). Together with time and initial conditions, they determine the behavior of the variables. The variables include the reaction rates and the concentrations of the metabolites internal to the pathway; they will change when system parameters are changed.

Traditionally MCA is concerned with stable steady states [291, 396]. The main question that is addressed in MCA is: to what extent does a change in the system's parameters affect the steady-state behavior of the variables? Essentially MCA is therefore a sensitivity analysis of stable steady states.

One can measure the effect of changes in the parameters (e.g. in the concentration of an enzyme) on the steady-state variables (e.g. growth rate or metabolite concentrations) without any understanding of the system. The Response Coefficient is then used to quantify the extent to which a parameter affects a variable. The response coefficient is defined as the relative change in a steady-state variable $X_j$, such as a flux or a concentration, caused by a relative change in a parameter $p$. Choosing relative changes rather than absolute ones makes this coefficient become dimensionless. For a more satisfying argument for using relative rather than absolute changes, see ref [159]. To determine a response coefficient, one should measure the steady-state variables one is interested in under some reference condition and compare it with the corresponding variables after a change in the parameter $p$. Mathematically, the response coefficient $R_{p}$ of variable $X_j$ to a change in parameter $p$ reads:

$$R_{p}^{X_j} = \left( \frac{\Delta X_j / X_j}{\Delta p / p} \right)_{\lim \Delta p \to 0} = \frac{\partial X_j / X_j}{\partial p / p} = \frac{\partial \ln X_j}{\partial \ln p}$$

(1.1)

(using the mathematical relation: $1/x \cdot dx = d\ln x$ to arrive at the right-hand term). The subscript of the response coefficient is used to indicate the parameter that is changed; the superscript refers to the variable that is evaluated.

The mathematics of the above formulation adds that if the change in $p$ is only small, then the response coefficient is the derivative of $X_j$ with respect to $p$. The advantage of such small
modulations is that second order effects remain negligible. Accordingly the use of derivatives enables the strict derivation of the laws of control of metabolic systems. Therefore, all coefficients within MCA are defined in terms of derivatives, and strictly speaking apply to small (infinitesimal) changes only. The use of derivatives also furnishes a graphical method to determine response coefficients. Plotting the logarithm of the steady-state variable against the logarithm of the parameter, the slope of the curve equals the response coefficient. The use of derivatives has also a drawback, as it is not always feasible to make a very small change in a parameter experimentally, or the resultant effect on the variable is not measurable with sufficient accuracy [97]. However, the standard experimental approach [134, 173, 175, 313, 371] is to modulate the parameter over a fairly wide range around the steady-state value of interest. This then allows a fairly accurate determination of the slope (i.e. the response coefficient) at the interesting steady state. Moreover, response coefficients can be used as good indicators of what happens in case of larger changes (cf. [339, 340] for elaborations on this point).

Successful attempts have been made to extend MCA into the domain of large changes. The first approach included second order derivatives, which enabled larger changes in parameters to be evaluated [157]. This requires more detailed kinetic information, and leads to mathematical equations that would make most molecular biologists, physiologists and biochemists feel miserable, and as such restricts its applications. Small and Kacser introduced the 'deviation index' [339], which can be used to predict the effect of a large change in a parameter on the flux. The same authors also developed a 'universal method' to increase a particular flux [177], and to increase a particular metabolite concentration [341] by making large changes in parameter values. (See also Westerhoff and Kell [395] for discussion on large changes and a similar strategy). Kholodenko et al [189] recently developed yet another strategy for designing large metabolic changes, but this was less based on MCA.

Having simply measured the response coefficient, one may wish to understand which factors determine the response of a metabolic system to a parameter change. The first important determinant will be the extent to which the activity of an enzyme is affected by the parameter. The magnitude of the initial, so-called local change in the activity of the enzyme that is affected by parameter \( p \), is described by the elasticity coefficient [54, 180]. In general elasticity is defined as the relative change in the activity of an enzyme, divided by the relative change in a parameter or variable that caused the activity to change. For a parameter change, the elasticity might, at first glance, seem the same as the corresponding response coefficient, but it is conceptually very different. The response coefficient describes the (small) change in the variables before and after a new steady state has been achieved, and is therefore evaluating global (or systemic) properties. The elasticity coefficient, however, evaluates the immediate effect of a change in a parameter or variable on the rate of an enzymatic reaction, without allowing the whole system to react. It quantifies the sensitivity of an enzyme as if in isolation and is
therefore a *local* property of that enzyme. Importantly, one still has to evaluate the elasticity of an enzyme towards a particular metabolite or parameter in the presence of the *other* metabolites and effectors at their steady-state levels. Elasticities are not constant properties of enzymes: other metabolites and effectors may alter the elasticity of an enzyme towards the concentration of some metabolite or parameter. For example, the concentration of ATP may affect the impact of a change in F26bP on the activity of PFK (see, e.g. [35, 344]). Likewise, the presence of intracellular glucose can affect the sensitivity of the transport step to extracellular glucose (chapter 4).

In mathematical terms, the elasticity coefficient of enzyme *i* with respect to parameter *p* reads:

\[
\varepsilon_p^i = \left( \frac{\partial \ln v_i}{\partial \ln p} \right)_{X_i}
\]  

(1.2)

where \( v_i \) is the activity of enzyme *i*. The derivatives are partial, i.e. the derivative is taken keeping all metabolite concentrations \( X_i \) that may affect the rate of enzyme *i* constant at their steady-state values.

The immediate effect of a change in parameter *p* is described by the elasticity coefficient of enzyme *i* to parameter *p*. The effect of that initial change in the activity of enzyme *i* on the system’s variables, after a new steady state is reached, is described by yet another coefficient: the Control Coefficient. The control coefficient is defined as the relative change in a steady-state variable \( X_j \) (such as the flux) divided by the relative change in the local activity of an enzyme \( v_i \) [192, 331]. Again, in mathematical terms:

\[
C_{i,j}^X = \left( \frac{\Delta X_j / X_j}{\Delta v_i / v_i} \right)_{\lim \Delta v_i \to 0} = \frac{\partial \ln X_j}{\partial \ln v_i} \frac{\partial v_i}{\partial v_i} \]  

(1.3)

To carry out an experiment to determine the control coefficient of enzyme *i* on the steady-state variable \( X_j \), one should change the activity of the enzyme by changing a parameter that *specifically* affects the enzyme (see section 1.4 on Tools in the study of metabolism). In the end, one *measures* the response of the system to the change in the parameter *p*. This response depends on the extent to which parameter *p* affects the activity of enzyme *i* (as quantified by the elasticity coefficient), and on the extent to which this change in activity affects the steady state variable \( X_j \) (as quantified by the control coefficient):
This equation shows the 'combined response property' of metabolic systems [159, 178]. By rearranging Eq. 1.4, the control coefficient is calculated from the measured response coefficient and elasticity (i.e., \( C=R/e \)). It should be noted that when a parameter affects more than one enzyme (e.g. temperature), the total response of the system will be the sum of the combined responses of all the individual enzymes [160, 182, 390]. This will be the basis of the derivation of the so-called connectivity theorems in the next section.

The combined response property allows regulation to be defined quantitatively. The elasticity of an enzyme has been referred to as its regulability [160], i.e. the extent to which an enzyme is sensitive to effectors, either external ones (e.g. hormones, nutrients, drugs) or internal ones (e.g. effectors, co-factors). The control coefficient can then be considered the regulatory capacity [160], i.e. the extent to which a change in the enzyme's activity (brought about by some effector) is able to affect a system variable, such as the flux or the concentration of a key intermediate. The combination of the two (the partial response coefficient of the enzyme, Eq. 1.4) has been referred to as the 'regulatory strength' of a regulatory mechanism, as it evaluates the impact of a modifier of an enzyme on a system variable [182].

Although the control coefficient is by far the best-known coefficient of MCA, it is conceptually the most difficult one (especially from the experimental point of view) because, as we have seen, one cannot measure a control coefficient directly. One can only change the activity of an enzyme by a parameter that (specifically) affects that activity, and we have defined such effects of parameters in terms of response coefficients. Why burden the scientific community with control coefficients, then? The problem with response coefficients is, that they depend on the parameter that was used to affect the activity of an enzyme. For example, a response coefficient of two inhibitors with respect to the flux will differ if their inhibitory constants are different. The control coefficient of an enzyme, however, is independent of which parameter was used to change its activity. Control coefficients really describe the contribution of each step in a pathway to the overall behavior of that pathway.

In their original paper, the Berlin group has defined control coefficients in terms of enzyme activities, as I have done here [144]. However, 'control coefficients' were defined in terms of enzyme concentrations by Kacser and co-workers [178, 180], and this definition is still in use (see, e.g. [98]). This is because in the ideal metabolic pathways that are studied or assumed in most metabolic control analyses [192, 197], the elasticity of a reaction rate towards the concentration of enzyme catalyzing that reaction equals 1 (i.e. the activity of an enzyme is proportional to its concentration). Under these circumstances, the response coefficient to that enzyme concentration will be the same as the control coefficient (see Eq. 1.4), and both
coefficients may be used. It has been proposed to specify this ‘control coefficient’ as an ‘enzyme concentration response coefficient’, because one measures the response of the system to a change in a parameter, i.e. the enzyme concentration (see also [307]). This becomes important in non-ideal cases where the rate of an enzyme-catalyzed reaction is not proportional to the concentration of the corresponding enzyme (e.g. in group transfer pathways and channeled pathways [190, 196, 369]). Essential theorems yet to come would appear to fail were the control coefficients to be defined in terms of enzyme concentrations rather than enzyme activities.

1.1.2 The theorems of MCA: how enzymes behave in a network

There is a number of laws that govern the control and regulation of metabolic pathways. These can be formulated precisely in terms of relationships between control coefficients, and between control coefficients and elasticities. Here we only give the relations and make them plausible by thought experiments. Mathematical proofs are in the literature to which we shall refer.

**Summation theorems**

When the activities of all $n$ enzymes that constitute a pathway are increased by the same factor, one should expect to see a new steady state with an increase in the flux $J$ by that same factor, the concentrations of the metabolites $M_j$ remaining constant [144, 178, 396]. In terms of control coefficients, this reads:

$$
\sum_{i=1}^{n} C_i^J = 1, \quad \text{and} \quad \sum_{i=1}^{n} C_i^{M_j} = 0
$$

(see [396] for mathematical proof via Euler’s theorem of homogeneous functions).

The flux-control-coefficient summation theorem indicates that there is a flux limitation, and it suggests that this limitation need not be conferred by a single *rate-limiting step*. In many theoretical and experimental studies the control on the flux was distributed among the enzymes that constituted the pathway (see, e.g. [50, 97, 133, 134, 257, 371]; Fell’s book [98] describes quite a few examples in detail). In the extreme case of equal distribution of flux control coefficients, each enzyme would have a control of about $1/n$, $n$ being the number of enzymes in the system. As (in a real cell) $n$ is large, each enzyme will have (very) little flux control. Kacser [179] reasoned along these lines to explain why most mutations are recessive, the rationale being that the reduction in enzyme level in a heterozygous was insufficient to exert a significant metabolic phenotype. Control may not always be quite that distributed (see e.g. chapter 7) and the occurrence of negative control coefficients (the control of an enzyme in one branch over the flux in a parallel branch is generally negative) causes total controllability (defined as the sum of
absolute values of the control coefficients) to exceed 1 substantially [181]. Yet the conclusion should be that the average control coefficient of an enzyme tends to be small.

**Connectivity theorems**

Slightly more complicated and less familiar, but extremely important, are the connectivity theorems of metabolic control. They allow control of fluxes and concentrations to be understood in terms of enzyme properties. To understand the relationships, we shall perform the following thought experiment: in a steady state, we instantaneously increase the concentration of metabolite $M$. This will affect the steady state only transiently, because no parameter was changed. Consider the flux through the system. The impact on the flux of the change in $M$ can be described by the 'combined response' that was discussed in the preceding section: $M$ affects the activities of the enzymes in the pathway (described by the elasticities), and these affect the flux (via the flux control coefficients). In the end, however, the total response of the system to a change in the concentration of $M$ should be zero [390]:

$$\sum_{i=1}^{n} C_i^J \varepsilon_i^M = 0 \quad (1.6)$$

The crux of the argument is that the total response of the system is the sum of all the responses of the individual enzymes and is zero for the steady-state condition when a variable is changed [182]. The equation demonstrates one of the connectivity theorems linking flux control coefficients to elasticities [178].

There are similar connectivity theorems for concentration control coefficients [390]. For all metabolite concentrations $M_j \neq M$, the same argument as for the flux applies, and similar connectivity theorems can be derived (replacing $J$ by $M_j$). When the response of the perturbed variable $M$ itself is evaluated, however, the response of the system should be such that the original amount of $M$ that was added, is removed. Hence:

$$\sum_{i=1}^{n} C_i^M \varepsilon_i^M = -1 \quad (1.7)$$

The combination of the theorems

Until now, we have defined coefficients that quantify the sensitivity of global, systemic properties such as fluxes and steady-state metabolite concentrations towards enzyme activities (control coefficients) on the one hand, and the sensitivity of local enzyme activities towards parameters and metabolite concentrations (elasticities) on the other. Furthermore, we have found relationships between these control coefficients and elasticities in the form of summation...
and connectivity theorems. We are now ready to do the most important and most satisfactory trick of MCA: \textit{Combining the summation theorems with the connectivity theorems, we are able to express all control coefficients in terms of the elasticities of the enzymes towards the internal metabolite concentrations.}

For a simple system with two enzymes and one metabolite coupling the two enzymatic steps (Fig. 1), there are four equations (two summation and two connectivity theorems) and four unknowns (flux and concentration control coefficients of enzyme 1 and 2). Such a set of equations can be solved, and the solutions for the four control coefficients read as follows:

\begin{align*}
C_1^J &= \frac{\varepsilon_2^X}{\varepsilon_2^X - \varepsilon_1^X}, \quad C_2^J = -\frac{\varepsilon_1^X}{\varepsilon_2^X - \varepsilon_1^X} \\
C_1^X &= \frac{1}{\varepsilon_2^X - \varepsilon_1^X}, \quad C_2^X = -\frac{1}{\varepsilon_2^X - \varepsilon_1^X}
\end{align*}

(1.8a-d)

This exercise shows the real power of MCA: one can understand the extent to which a certain enzyme controls the flux or a metabolite concentration on the basis of limited kinetic information concerning the enzymes in the pathway. Note that the elasticities of both enzymes appear in the control coefficients for this two-enzyme pathway. This demonstrates that (control of) flux and concentration are determined by the whole system, and are therefore \textit{systemic} properties.

Highly regulated enzymes are expected to have high elasticities (this is a way of defining regulability [160, 390]), and although they will be recognized as 'key-enzymes' by many, they tend to have little control on the flux (cf. Eq. 1.8). Phosphofructokinase (PFK) is an appropriate example. Biochemical textbooks tend to designate PFK as the key enzyme or "pacemaker" of glycolysis (e.g., [350]), controlling the flux of that pathway. However, attempts to increase the glycolytic flux by overexpression of PFK have failed [81, 319]. This may have been because the higher amount of enzyme was less active due to a down-regulation of fructose 2,6-bisphosphate (F26bP) [81], a strong activator of PFK. Also in a \textit{pfk26/pfk27} double deletion mutant, not able to synthesize F26bP, the flux was not affected because of increased levels of fructose 6-phosphate.
(F6P), the substrate of PFK [45]. These examples serve to illustrate that it is impossible to decide whether PFK is a pacemaker enzyme based on the kinetic properties of that enzyme alone. If one were to make a prediction, however, the extensive regulation of the enzyme should make PFK a poor, rather than a good, candidate for having a high flux control coefficient. This is a particularly nice example where false traditional biochemical explanations have been debunked by MCA [98].

1.1.3 Large systems and other complications in (experimental) control analysis.

Large metabolic systems

Metabolic systems of only two enzymes do not appear very relevant when facing the complexity of metabolic networks. The preceding treatment of MCA forms only the basis from which extensions can be made to tackle more realistic metabolic systems. For a system of any size, the summation and connectivity theorems are still valid. Together with additional theorems, their total number remains equal to the number of control coefficients. Large systems can therefore be solved in essentially the same way as the simple system of the preceding paragraph, although the mathematics becomes more elaborate. The power of linear algebra has been enlisted to deal with control analysis of systems of arbitrary size [291]. Several matrix notations have been introduced that relate a matrix with all control coefficients (the control matrix C) to a matrix with all elasticity coefficients (the elasticity matrix E) [161, 291, 316, 338, 392, 394]. By arranging the coefficients in the matrices in a proper way, a very elegant relationship can be obtained:

\[ C = E^{-1} \]  \hspace{1cm} (1.9)

Thus, the control coefficients can be calculated if all the elasticities are known by taking the inverse of the elasticity matrix. Also the reverse is possible [392].

Important complications arise from branches and moiety conservation. Moiety conservation is the phenomenon that some chemical entity is cyclically transferred from one compound to another, transforming the compounds but leaving the basic chemical moiety shared by the compounds intact. Examples are the adenine nucleotides, where phosphate is transferred between AMP, ADP and ATP, and NADH and NAD, where electrons (and a proton) are exchanged. The result of branches and moiety conservation is that some of the metabolic variables become dependent on one another and hence, that the number of independent relationships is less than the number of unknown control coefficients [99, 163]. This problem can be tackled by defining the elasticity coefficients in terms of the truly independent variables [396]. Alternatively, the control and elasticity matrices are reduced to describe the coefficients with respect to some of the dependent variables only (making this set independent). The latter approach requires additional matrices that describe the relationship between the dependent variables.
variables and the independent ones (the Link matrix \( L \) for moiety conservations in metabolites and the Kernel matrix \( K \) for the relations between steady-state fluxes) [291]. It is, however, more suitable than the former approach for more complex systems in which moiety conservation relationships are less obvious.

Thus, control analysis (for stable steady states) is possible for any (ideal) metabolic system of arbitrary size and complexity. The experimental determination of control coefficients, however, is rather laborious, and a complete experimental control analysis of a metabolic system is a heroic task which has been performed for only a few relatively small systems, such as mitochondrial respiration [134, 138], gluconeogenesis in rat hepatocytes [133] and the yeast tryptophan biosynthetic pathway [257]. Also not all enzymes may be amenable to experimental modulation. Therefore, methods have been developed to simplify metabolic pathways by grouping enzymes in modules (also called blocks or simply groups) and to treat those modules as single “super enzymes”. These approaches have been called ‘global’ [396] or ‘modular’ control analysis by Westerhoff and co-workers [332] or ‘top-down control analysis’ by Brand and colleagues [50]. Modules with single degrees of freedom have been called ‘monofunctional units’, and have been strictly defined by Rohwer et al [309]. These units have particularly useful properties that will be used in chapter 7. Monofunctional units will be discussed in more detail shortly.

**Other complications: hierarchical control**

Although large systems have their specific problems in control analysis, there are a few other, more fundamental, phenomena that may complicate control analysis even in relatively simple systems. One of these is the role of regulation of gene expression. The effect of a change in the activity of an enzyme may have some direct metabolic effect, but may also indirectly affect the expression of other enzymes. An experimental example is the work by Jensen and coworkers on the \( \mathrm{H}^+\)-ATPase in \( \mathrm{E. coli} \) [172, 173]. When they modulated the activity of the \( \mathrm{H}^+\)-ATPase, they found a control coefficient for growth rate of 0.0, i.e. no effect on growth rate. It turned out that \( \mathrm{H}^+\)-ATPase had a negative control on the rate of respiration, so that an increase of \( \mathrm{H}^+\)-ATPase activity was balanced by a decrease in respiration, a phenomenon they called ‘inverse respiratory control’ [174]. These effects can be included in control analysis by resorting to ‘hierarchical control analysis’ as developed by Westerhoff et al. [181, 393].

Hierarchical control may also be expected when isoenzymes are present that are expressed only under specific growth conditions. The family of hexose transporters in yeast, the \( \text{HXT} \)-gene family [207], may be a good example. Some enzymes are induced by glucose, whereas others are repressed [271]. Moreover, the hexose transporters themselves have been implicated in glucose-induced signaling [292, 382]. When one of the isoenzymes is deleted, therefore, this may affect the expression of the other genes. Accordingly, \( \text{HXT1} \) encodes a low-
affinity glucose carrier [292, 293] that is glucose induced [271], but its deletion affected high affinity transport as well [219]. Hierarchical effects are expected when a genetic approach is used to alter the activity of a particular enzyme (see section 1.4). The time scale at which the alteration in enzyme activity is effective is long (e.g. during growth of the genetically modified cells) and the cells may have time to respond to the alteration. Perturbation at a much shorter time scale, such as the addition of specific inhibitors, should allow the determination of the subsequent metabolic impact before hierarchical effects become significant.

Other complications: co-response analysis

Another complication with experimental control analysis is the need for the exact quantification of the elasticity of the enzyme with respect to the parameter that was used to specifically modify the enzyme's activity (cf. Eq. 1.4). As will be amply illustrated in chapter 5, the determination of these elasticities is not always straightforward (or accurate [337]). Hofmeyr and Cornish-Bowden have developed an alternative approach to determine the control coefficients without the need for exact quantification of the extent to which the enzyme's activity was modulated. Their approach is based on co-response analysis [161, 162]. A co-response coefficient \( \Omega \) is defined as the ratio of two response coefficients:

\[
\Omega_{ij} = \frac{R_{ij}^{X}}{R_{ij}^{X_j}} = \frac{\partial \ln X_i}{\partial \ln X_j}
\]

The co-response coefficient describes the relative response of two variables to a change in a single system parameter. It can be a measure of 'external regulation', i.e. the relative regulation of two metabolic variables by an external parameter (see also section 1.2). If, in the two-enzyme system of Fig. 1, the parameter \( p \) affects enzyme (or module) 1, the co-response of the flux through enzyme (or module) 2 and the concentration of \( X \) indicates the extent to which parameter \( p \) can regulate the flux through enzyme 2 via metabolite \( X \). In such a case, this co-response coefficient is equal to the (block) elasticity of enzyme (module) 2 with respect to metabolite \( X \) [159]. Importantly, knowledge of the matrix of co-responses of a metabolic system under study allows a complete control analysis of the system (i.e., calculation of all control and elasticity coefficients). What is required to obtain all necessary co-response coefficients, is a way to modulate the activities of the enzymes in specific ways and to measure the concentrations of metabolites and the fluxes through the system [161, 162]. Co-response coefficients are used in chapter 5 to compare effects of different parameter changes on the glycolytic flux and on the frequency of glycolytic oscillations.
Enzymes that can be grouped into monofunctional units, have the important property that they exhibit identical co-response coefficients for metabolic variables outside the unit [194, 309]. This reduces the number of perturbations that is required for a full co-response analysis [161]. The properties of monofunctional units will be used in chapter 7. They may also be useful in a strategy towards the elucidation of the function of unknown genes identified by the genome sequencing projects [264, 266, 356]. The rules for delineating monofunctional units are: i) there should be only one independent flux linking the unit and the rest of metabolism, ii) there should be no metabolites inside the unit that directly affect the rate of any enzyme outside the unit, and iii) there should be no metabolites in the unit that are part of a moiety-conserved sum outside the unit [309].

In short, the unit should behave as if it were an enzyme catalyzing a (completely coupled) reaction. Two simple enzymes in a chain of reactions will usually fulfill these conditions (e.g. phosphoglycerate mutase and enolase most likely behave as a monofunctional unit). Larger groups of enzymes may also be grouped (e.g. glycolysis as a whole, considering the adenine nucleotides and the redox couple NADH/NAD$^+$ as to reside outside the unit; see ref [307, 309] and chapter 7 for discussion).

Other complications: non-ideal metabolism

In all treatments above metabolism has been treated as ideal, i.e. enzyme activity being proportional to enzyme concentration and independent of the concentration of other enzymes [192]. This may not be the case under conditions that prevail in the cell. Effects such as macromolecular crowding, enhancing enzyme-enzyme interactions and possibly channeling [348], and high enzyme concentrations comparable to the concentrations of conserved moieties, may invalidate these presumptions of ideality [193]. Also group-transfer systems, such as the bacterial phosphotransferase system (PTS [280]), deviate from the ideal case [193, 308]. The result that the sum of enzyme-concentration based control coefficients (i.e. response coefficients with respect to enzyme concentration) can vary between 1 and 2 in the phosphotransferase system [308, 369], shows that non-ideality can lead to intensified control by gene expression. Non-ideal metabolism requires control coefficients to be defined in terms of elementary steps in order for the classical theorems of control analysis to be still valid [192, 193, 197].

1.2 Analysis of the stability of steady states and the conditions for oscillations in metabolic systems

1.2.1 Stability analysis of steady states

Control analysis deals with stable steady states. This implies that steady states can be unstable as well. Indeed, a large part of this thesis analyzes glycolysis under conditions where
the system has become unstable and starts to oscillate around the unstable steady state. In this section we briefly outline the conditions under which steady states may become unstable, and the way this can be analyzed. The mathematical techniques for studying the stability of steady states and the resulting dynamic behavior of variables are often referred to as (nonlinear) System Theory (or 'stability analysis') [156, 351].

The time dependence of metabolite concentrations can be described as a function of the rate at which they are produced, the rate at which they are consumed and the rate at which they are transported. For the system of Fig. 1, this reads:

$$\frac{d[X]}{dt} = v_1 - v_2 = f_1(X, p_1) - f_2(X, p_2) = f(X, p_1, p_2)$$  \hspace{1cm} (1.11)

where the right-hand terms stress the fact that the rates of step 1 and 2 are functions of the concentration of X and of some kinetic parameters $p_1$ and $p_2$ of enzyme 1 and 2, respectively. A steady state is obtained when $v_1 = v_2$, such that the concentration of X is constant in time (at $[X]_{ss}$) and the flux through the system is $J = v_1 = v_2$. To test whether the system is stable with respect to a (small) change in [X] one should solve Eq 1.11 for what happens when [X] is slightly perturbed from its steady-state value. Introducing the deviation from steady state $x = [X] - [X]_{ss}$, Eq. 1.11 can be rewritten as:

$$\frac{dx}{dt} = f(x, p) \approx \left(\frac{\partial f(x, p)}{\partial x}\right)_{x=0} \cdot x = \lambda \cdot x$$  \hspace{1cm} (1.12)

The equation is based on the first-order Taylor expansion of $f(x, p)$ around steady state ($x=0$), which is valid for small $x$ only (otherwise higher-order terms should be taken into account). $\lambda = (\partial f/\partial x)_{x=0}$ is the so called eigenvalue of the system, which is central to System Theory. The eigenvalue is related to the unscaled elasticity coefficients and the stoichiometric structure of the system [397]:

$$\lambda = \frac{\partial f}{\partial x} = \frac{\partial f_1}{\partial x} - \frac{\partial f_2}{\partial x} = \tilde{\varepsilon}_x^1 - \tilde{\varepsilon}_x^2$$  \hspace{1cm} (1.13)

A tilde (~) is used to denote unscaled elasticity coefficients [307]. The eigenvalue determines the stability of the system, since the well-known solution of Eq. 1.12 is:

$$x(t) = x(0) \cdot e^{\lambda t}$$  \hspace{1cm} (1.14)
If $\lambda < 0$, the system will relax back to the original steady state (if $t$ goes to infinity, the deviation from steady state, $x$, goes to zero) and the system is stable. If $\lambda > 0$, however, a fluctuation in $[X]$ and hence, a nonzero value for $x(0)$, will lead to an exponential increase in $x$: the system is unstable, at least locally.

Stability analysis for a system with only a single independent metabolite (Fig. 1) can be well illustrated by plotting the rate characteristics of the reactions with respect to the concentration of the linking metabolite $X$ (Fig. 2). Such an analysis is an important ingredient of chapter 6. In Fig. 2A, the activity of enzyme 1 was taken to be insensitive to $[X]$, whereas enzyme 2 was taken to be substrate inhibited (a rare form of kinetics that is seen for, e.g., phosphofructokinase with respect to its substrate ATP). Two intersection points are found, indicating the existence of two so-called fixed points that the system can attain. The fixed point at low $[X]$ is stable: an increase in $[X]$ would lead to a higher consumption than production of $X$ ($v_2 > v_1$) and $[X]$ would decrease again, and vice versa. In this steady state, $\lambda = (\partial f/\partial x)_{x=0} < 0$, since $f = v_1 - v_2$, $(\partial v_i/\partial x) = 0$ and hence $(\partial f/\partial x) = -(\partial v_i/\partial x) < 0$. The fixed point at high $[X]$, however, is unstable: an increase in $[X]$ would lead to an increase in $[X]$ as $v_1 > v_2$ in that case. A decrease in $[X]$ would bring the system to the steady state at low $[X]$. Indeed, here $(\partial f/\partial x) = -(\partial v_i/\partial x) > 0$. This fixed point is sometimes called an unstable steady state.

![Figure 2. Examples of possible rate characteristics of the enzymes of the system depicted in Fig. 1 with respect to the concentration of the linking metabolite $X$.](image)

In Figure 2B the rate of enzyme 1 is stimulated by its product (but inhibited at sufficiently high concentrations), whereas enzyme 2 exhibits 'normal' Michaelis-Menten behavior. In this case, three fixed points are observed, the middle of which is unstable. The other two fixed points are stable steady states. The steady-state behavior of the system is now determined by the initial conditions (or history of the system). If the initial concentration of $X$ were high, the high $[X]$, will
be reached, but if [X] were low initially, the low [X] will be reached. Moreover, a large change in [X] can now force the system from one stable steady state to the other stable steady state: the system displays switch-type behavior, known as hysteresis (see [43] for a good example).

1.2.2 Stability analysis and oscillations

As was seen from Fig. 2, "odd" kinetics, such as substrate inhibition or product activation (also seen for phosphofructokinase with ADP (and especially AMP) as the product [28, 35, 217, 260, 295, 344]) have the potential to introduce instability to a system. To understand more complex behavior such as oscillations or chaos, more independent metabolites are required [156]. Although the introduction of more variables makes the mathematics more complicated and leads to more eigenvalues, the basics are the same as for one variable. Thus, a 2-variable system may be described by:

\[
\begin{align*}
\frac{dx_1}{dt} &= f_1(x_1, x_2, p) \\
\frac{dx_2}{dt} &= f_2(x_1, x_2, p)
\end{align*}
\]

(1.15)

In this case the two differential equations are coupled (both functions \( f \) may depend on \( x_1 \) and \( x_2 \)). The solutions to the first order terms of the Taylor expansion of this set of equations are:

\[
\begin{align*}
x_1(t) &= a_1 \cdot e^{\lambda_1 t} + b_1 \cdot e^{\lambda_2 t} + c_1 \\
x_2(t) &= a_2 \cdot e^{\lambda_1 t} + b_2 \cdot e^{\lambda_2 t} + c_2
\end{align*}
\]

(1.16)

where \( a, b \) and \( c \) are constants that depend on the initial conditions. There now are two eigenvalues, \( \lambda_1 \) and \( \lambda_2 \), and only one of them needs to be positive for the system to be unstable. The eigenvalues can become complex. Complex eigenvalues can lead to oscillations, which follows from the definition of complex exponentials:

\[ e^{\lambda t} = e^{\Re \lambda t} \cdot \left( \cos(\Im \lambda \cdot t) + i \cdot \sin(\Im \lambda \cdot t) \right) \]

(1.17)

where \( \Re \lambda \) and \( \Im \lambda \) are the real and imaginary parts of \( \lambda \), respectively. Clearly, \( \Im \lambda \) corresponds to the angular frequency of the oscillatory component, denoted by \( \omega \) in this thesis (see section 1.3.3 and chapter 2). Because complex eigenvalues always occur in pairs with equal real parts but opposite imaginary parts, Eq. 1.16 typically reduces to:
Figure 3. The glycolytic pathway of Saccharomyces cerevisiae. The alcoholic fermentation branch is included. Dashed lines indicate regulatory interactions.

\[
x_1(t) = d_1 \cdot e^{Re\lambda t} \cdot \cos(\omega t + \phi_1) \\
x_2(t) = d_2 \cdot e^{Re\lambda t} \cdot \cos(\omega t + \phi_2)
\]

where \( \phi_1 \) and \( \phi_2 \) denote the phases of the oscillatory component. Thus, a system that is characterized by eigenvalues with negative real parts and nonzero imaginary parts will exhibit oscillatory relaxation back to the steady state (damped oscillations). The damping factor depends on the real part of the eigenvalues; the frequency depends on the imaginary part of the
eigenvalues. A system with positive real parts and nonzero imaginary parts, however, will oscillate away from the unstable fixed point and may settle into a so-called limit cycle. A limit cycle is a state in which the metabolites oscillate with constant frequency and amplitude around their unstable steady-state concentration. Plotting the two metabolites in time against each other, the resulting phase plane will show a closed curve with the unstable steady state inside (which explains the name).

Since the eigenvalues of the system depend on the kinetics of the system, there can be critical changes in the parameters where eigenvalues change sign or become complex: such sudden changes in eigenvalues and the resulting changes in qualitative behavior of the system are called bifurcations. An important bifurcation where, upon a change in some parameter, the real part of one of the complex eigenvalues becomes positive, is called the Hopf bifurcation [124, 256]. At this bifurcation, limit cycles may set in. Thus, periodic phenomena in biology can be understood from Systems Theory as systems that have moved beyond the Hopf bifurcation. This also explains why oscillations are only found under some specific conditions (see also section 1.3.3 and chapter 2).

1.3 Glycolysis of yeast: facts and open questions

The model system used throughout this thesis is the glycolytic pathway of the Baker's yeast *Saccharomyces cerevisiae* (Fig. 3). It is probably the most intensively studied system of biochemistry, undoubtedly spurred by biotechnological interests in valuable products such as ethanol, carbon dioxide and yeast biomass. The research on (yeast) glycolysis has passed through a few distinct phases of interest. In the first half of this century, components that constitute the pathway were identified and studied. The physiology of glycolysis and mechanisms for phenomena such as the Pasteur effect were important issues. The Pasteur effect is the observation that, at moderate glucose concentrations, anaerobic glycolysis proceeds much faster than aerobic glycolysis [102, 281, 372].

In the late fifties, early sixties, fluorescence measurements on intact cells showed damped oscillations in NADH [67, 89], induced by the same aerobic-anaerobic transitions as were used for studying the Pasteur effect. This observation led to intensive studies over the subsequent two decades on the mechanism of glycolytic oscillations (section 1.3.3) which coincided with the study of the kinetic characteristics of the glycolytic enzymes (as described in chapter 8). The increased focus on the genetics of yeast and the hunt for genes encoding cell components led to increased knowledge of the molecular biology of the glycolytic enzymes and their regulation (see for review [102]). The large number of tools to study and modify yeast genetically has transformed this yeast into the model for studying the molecular basis of a eucaryotic organism.
In recent years efforts have focused on the identification of the components of the signaling pathways in yeast, including signals that regulate the expression of glycolytic enzymes [66].

The study of yeast (anaerobic) glycolysis pursued in this thesis deals mainly with regulation and control at the metabolic level. Thus at a given physiological state, the behavior of glycolysis is studied and attempts have been made to understand this behavior on the basis of the kinetic properties of the enzymes and their interactions. It may seem that most questions about the metabolic control and regulation of this extensively studied pathway have been answered. This is not the case (as already illustrated for phosphofructokinase). In this section I highlight those facts from the extensive literature on yeast glycolysis that suggest that the regulation and control of glycolysis is still not completely understood. Most of the corresponding issues are addressed in this thesis.

1.3.1 Regulation of three ‘key enzymes’ in glycolysis: hexose transport, hexokinase and phosphofructokinase

The glycolytic pathway begins with the transport of hexoses across the plasma membrane. Carriers that transport glucose also transport fructose and mannose, although with different affinities ([36, 209], see also chapter 5). After sequencing the genome of *S. cerevisiae*, it turned out that there is a family of homologous sugar transporters with 20 members: *HXT1*-17, *GAL2*, *SNF3* and *RGT2*. The latter two have been identified as glucose sensors, rather than glucose transporters [208, 270]. *GAL2* is the galactose permease, which can also transport glucose [46, 207]. Of the *HXT*-genes, only *HXT1*-4 and *HXT6* and *HXT7* seem physiologically relevant, as a strain deleted in all these transporters (but not in the other *HXT*s) fails to grow on glucose [292, 293]. The role of the other *HXT*s is unclear.

The hexose transporters have different affinities for glucose. *HXT1* and *HXT3* have a low affinity for glucose (50-100 mM), *HXT6* and *HXT7* have a high affinity for glucose (1-2 mM) and *HXT2* and *HXT4* have intermediate affinities [292]. The mRNA levels of the individual *HXT*s fit qualitatively with the apparent kinetics of glucose transport during batch growth [M. Walsh, personal communication] and continuous cultivation [Kruckeberg, Schepper, Diderich, personal communication]. Thus, during early exponential growth on glucose, low affinity transport kinetics is paralleled by predominant expression of *HXT1*, whereas at glucose depletion high affinity glucose transport is observed, accompanied by high *HXT7* expression. Interestingly, during this affinity modulation the maximal rate of transport remained constant [383]. This observation begs the question why yeast requires low affinity carriers if it has high-affinity carriers with similar capacity at its disposal.

The mechanism of glucose transport has been a matter of debate, but there seems to be a consensus that hexose transport in *S. cerevisiae* occurs via facilitated diffusion [36, 209]. The controversy was about the involvement of hexose phosphorylating enzymes in high affinity transport [37, 213]. The fact that high affinity transport was abolished in a ‘triple kinase mutant’
(a mutant lacking hexokinase P1 and PII and glucokinase [37]), was later explained to arise from insufficient time resolution of the uptake assay [343]. A similar conclusion was drawn by Fuhrmann [106] on the basis of kinetic studies in vesicles isolated from triple kinase mutants. These authors also demonstrated counterflow of labeled glucose, a phenomenon characteristic of facilitated diffusion carriers (or in fact for enzymes with one substrate and one product in which the mechanism involves two forms of unbound enzyme [76], as found in certain isomerases [100]). The counterflow experiments of Fuhrmann et al. and the demonstration of ‘normal’ high-affinity transport in a kinaseless mutant [343] form the strongest evidence for a facilitated diffusion mechanism to date.

What remains unclear is the regulation of the activity of the transporter. Effects of ATP [328] and glucose 6-phosphate [12, 108, 306] have been reported, but regulation by the latter has been disputed [274]. Complex formation of the transporter with other cell-components has been suggested, but evidence is lacking [362, 363, 367]. The mechanism by which metabolism feeds back on the transporter is important for understanding the role of hexose transport in regulation and control of glycolysis. This prompted us to investigate the role of the ignored, but most likely candidate, intracellular glucose (chapter 5).

In the early eighties the biochemical community was surprised by the identification of a new regulatory metabolite in glycolysis: fructose 2,6-bisphosphate [373, 375]. This metabolite is a strong activator of yeast phosphofructokinase [18], the supposed key enzyme in glycolysis, and an inhibitor of fructose 1,6-bisphosphatase [374]. F26bP metabolism in yeast involves two separate enzymes, 6-phosphofructo-2-kinase (PFK-2) and fructose 2,6-bisphosphatase (F26bPase), in contrast to the mammalian case where a single enzyme carries out both functions [201]. PFK-2 is encoded by the genes PFK26 and PFK27 [45], which are regulated kinetically, post-translationally and transcriptionally [49, 104]. F26bPase is encoded by FBP26 [202]. More than one protein with F26bPase activity have been purified, exhibiting high and low affinity for F26bP [105, 185].

The complex regulation of F26bP metabolism and its pronounced effect on the activities of PFK and FBPase suggest an important role of this metabolite on the regulation of glycolysis. Recent results on yeast strains with altered fructose 2,6-bisphosphate metabolism, however, shed doubt on the precise role of this regulator. Yeast cells deleted in both phosphofructo2-kinases PFK26 and PFK27 and hence without any fructose 2,6-bisphosphate showed no increase in generation time [45]. They did show a longer transition time after transfer from ethanol to glucose containing medium [45]. These results are in line with what was observed after the introduction of heterologous PFK from Dictyostelium discoideum [96], which is not regulated by F26bP, and after site directed mutation of yeast PFK [141], rendering the enzyme insensitive to F26bP. In all three cases the rate of glycolysis was not affected, but rather large changes in metabolite concentrations were observed that appeared to have counteracted the modulation.
The levels of fructose 6-phosphate had increased, whereas those of fructose 1,6-bisphosphate and ATP had decreased. On the other hand, when PFK was overexpressed, decreased levels in F26bP were found, and no increase in the glycolytic flux was observed [81].

The role of F26bP in preventing futile cycling between PFK and FBPase [251] was questioned by the observations that in mutants with overexpressed FBPase activity and lacking F26bP, growth rate on glucose and the rate of ethanol production were hardly affected [44, 248]. Moreover, in a strain with high levels of F26bP under gluconeogenic conditions, growth rate on ethanol and glycogen content were the same [44, 248]. Consequently the lack of impact of F26bP on glycolysis appears to be in sharp contrast to its complex metabolism.

In the early nineties, the yeast community was perhaps even more surprised by yet another type of regulation in glycolysis, when it was found that a number of mutants that suffered from glucose toxicity were allelic and that the mutations were in the gene encoding trehalose 6-phosphate synthase (Tps1) [367]. The gene had been previously cloned independently by Gancedo's group [129] and by groups interested in trehalose metabolism [22, 380]. Tps1 catalyzes the condensation of UDP-glucose and glucose 6-phosphate to form trehalose 6-phosphate, a precursor for the disaccharide trehalose. In an as yet incompletely understood way, Tps1 prevents accumulation of hexose phosphates, especially fructose 1,6-bisphosphate, which occurs in a tps1Δ mutant at the cost of phosphate depletion and at a low ATP concentration [38, 165, 167, 227, 363]. Tps1-mutants do not grow on glucose but are killed by it, perhaps due to toxicity of the high levels of sugar phosphate or due to phosphate depletion.

It has become clear that Tps1 acts to reduce the activity of the first steps in glycolysis somehow. Complex formation of hexokinase, the glucose transporter and Tps1 has been proposed [363]. Yet, a direct mechanism of inhibition was suggested by the observation that trehalose 6-phosphate (Tre6P), the product of Tps1, competitively inhibited hexokinase in vitro [38]. The inhibitory constant for Tre6P was found to be 0.04 mM for hexokinase PII and 0.2 mM for hexokinase PI, with glucose as the competing substrate [38]. Hohmann et al. [165] found higher K_i-values (0.1 mM and 1 mM for HK PII and PI, respectively). Glucokinase does not appear to be inhibited by Tre6P. The concentration of Tre6P after glucose addition to cells grown on glycerol/galactose spiked to levels of about 1 mM [165], and the inhibition of hexokinase by the compound may therefore be significant. This of course also depends on the concentration of internal glucose and the affinity of hexokinase for glucose, matters that are addressed in chapters 4 and 8, respectively. On the other hand, a 50-fold overexpression of hexokinase did not lead to a tps1Δ phenotype, and this was taken as an argument against Tre6P inhibition alone being able to regulate the hexokinase activity [95]. Unfortunately, however, no Tre6P levels were measured in this study, which would allow a comparison between the Tre6P level and the inhibition of HK that is required.
An observation that appears to support the Tre6P-inhibition model is that mutants deleted in phosphoglucose isomerase (PGI) do not grow on fructose alone, but display a phenotype similar to that of \textit{tpslA} mutants, when grown on glucose or fructose [49, 102]. \textit{Pgi-}deletants are unable to synthesize glucose 6-phosphate from fructose 6-phosphate and hence, lack trehalose 6-phosphate when grown on fructose alone. The accumulation of sugar phosphates correlated with the activity of PGI [Eckhard Boles, personal communication]. \textit{Pgi-}deletants do grow on a fructose medium supplemented with small amounts of glucose. This suggests that Tpsl cannot regulate the hexokinase activity itself, but requires the presence of substrate. It cannot be ruled out, however, that binding of substrate (or product) leads to altered binding properties of Tpsl for hexokinase and/or the glucose transporter, but experimental evidence for such a mechanism is lacking so far.

Although the mechanism for Tpsl-mediated feedback is a matter of continuing debate, the need for an extensive feedback mechanism on hexokinase, other than ‘simple’ product inhibition of glucose 6-phosphate found in many other organisms, has not been addressed. This will be the focus of chapters 6 and 7.

\textbf{1.3.2 The control of the glycolytic flux}

The question of which steps in glycolysis control the glycolytic flux is long-standing, and obviously important for those industries that use yeast for the production of ethanol or carbon dioxide. Traditionally the irreversible steps in glycolysis, the kinases HK, PFK and PYK were considered important control points, with GAPDH and PGK as interesting outsiders. Reactions far from equilibrium indeed have a higher potential to be rate-controlling than reactions near equilibrium [159, 396]. The main argument for those enzymes being important controlling steps, however, that of complex allosteric regulation, has been falsified by MCA in terms of having less to do with control of flux than with homeostasis of metabolite concentrations (see above and [98, 160, 182]). Indeed, overexpression of these enzymes did not lead to an increased glycolytic flux [81, 319]. It should be noted, however, that the overexpression of one enzyme may affect the level of expression of other enzymes [393], as demonstrated for glycolytic enzymes in yeast [311]. This may be via regulation of gene expression by glycolytic metabolites [47, 48] and possibly via the protein burden [346]. The classical paper by Schaaff et al., where almost all enzymes of glycolysis were individually (or in pairs) overexpressed, did not consider such side effects that may complicate the interpretation of their results [319]. In any case, the control of the glycolytic enzymes on the flux through glycolysis does not appear to be large under the conditions studied so far, i.e. under glucose excess. If control does not reside at the glycolytic enzymes themselves, candidates for flux control are glucose transport, biosynthetic pathways and (other) ATP-consuming reactions.

Glucose transport has been implicated in many studies to have significant control over the glycolytic flux. An important argument has been that the intracellular glucose concentration
was thought to be negligible compared to the extracellular glucose concentration [21, 111]. This was rationalized by the much higher activity of the glucose phosphorylating enzymes as compared to the total activity of glucose transport, and the high affinity of those enzymes for glucose (about 0.1 mM [222]). This argument is not conclusive, however, as will be explained in chapter 4.

The observation that the decrease in glycolytic flux in a \textit{cdc35} temperature-sensitive mutant correlated with the activity of transport of the glucose analogue 2-deoxyglucose, was also taken as indicative for high flux control of the glucose transporters [263]. Although it was checked that other glycolytic enzymes did not change significantly, defects in cAMP-mediated signaling caused by the mutation in \textit{cdc35} are likely to have pleiotropic effects, which seriously complicate the interpretation of the results (see also chapter 5 for similar interpretation problems).

Another line of evidence comes from mathematical models of yeast glycolysis. All models published so far predicted significant control in the glucose transporter [77, 78, 108, 324]. Regulatory feedback on glucose transport was modeled, however, via glucose 6-phosphate and most of the models do not consider intracellular glucose as a possible feedback inhibitor of glucose transport. G6P-mediated regulation of glucose transport, however, is still controversial (see above). Chapters 4 and 5 are devoted to the regulation of the activity of the glucose transporter and to a reassessment of the control exerted by glucose transport on the glycolytic flux in resting yeast cells. This knowledge is subsequently used in a mathematical model of yeast glycolysis in chapter 8.

### 1.3.3 Glycolytic oscillations in populations of intact yeast cells

In the late fifties and early sixties oscillations in suspensions of yeast cells were observed when the fluorescence of NADH was followed after transitions from aerobic to anaerobic conditions. These oscillations were amongst the first biological systems that showed such oscillatory behavior. Although its physiological role was not understood and is still unclear to date, glycolytic oscillations received a lot of attention in the next two decades, especially by the groups of Chance and Hess.

We have studied glycolytic oscillations for two reasons. First, they serve as a model system to study the control properties of oscillatory systems. A control analysis of oscillatory systems is still at its infancy (see below). Second, dynamic states are much richer in information than single steady states. Dynamic behavior may yield valuable information regarding the regulatory interactions in glycolysis (see e.g. [299]). Dynamic behavior of glycolysis was used by Reuss and coworkers [305] to fit kinetic parameters for their glycolytic model. Glycolytic oscillations show limit-cycle behavior, which is much less sensitive to initial conditions than are transients (see below). Limit-cycle oscillations are therefore ideal for model validation.
The mechanism of glycolytic oscillations

Oscillations in all glycolytic intermediates, including its co-factors, have been found [31, 33]. Also the redox state and the energy state of the cells oscillated significantly. In more recent studies on intact cells it was found that the intermediates of the lower part of glycolysis, from glyceraldehyde 3-phosphate (GAP) and dihydroxyacetone phosphate (DHAP) until pyruvate (PYR) did not oscillate significantly, although the co-factors did [299, 300]. This difference may be due to the conditions applied and may be strain dependent. In the studies by Richard et al. [299, 300] the conditions were optimized to obtain sustained limit-cycle oscillations (see also [151, 283]). Many older studies on intact cells were done under conditions where only damped oscillations were observed.

Glycolytic oscillations have been reviewed regularly [125, 149, 150, 153, 365, 404] and recently a book appeared by Goldbeter, about biological rhythms, that gives many details on glycolytic oscillations in yeast [124]. The mechanism of the oscillations was studied mainly in cell-free extracts of yeast. A substrate injection technique was developed that allowed a continuous addition of substrate at a controlled rate. Oscillatory behavior was observed only within a specific range of the substrate injection rates [152]. Addition of glucose, G6P or F6P led to oscillatory behavior, but addition of F16bP did not [42, 152]. In this way it was suggested that phosphofructokinase is the enzyme indispensable for the occurrence of oscillations in glycolysis, and it was given a special name: the oscillophore [150]. This spurred the investigation of the kinetics of PFK, in order to understand how PFK could be responsible for the oscillatory behavior. PFK is substrate inhibited by ATP and indirectly product stimulated by AMP, via the action of adenylate kinase [28, 35, 217]. Such regulatory mechanisms have the potential to cause instability of the system (section 1.2). A mathematical model of glycolysis, based on the kinetic properties of PFK only, was able to oscillate and could fit the experimental observations in yeast cell-free extracts surprisingly well [40, 122, 124, 126]. There is therefore little doubt about the central role of PFK in the generation of oscillations in glycolysis.

Alternative explanations do exist, however. One stressed the possibility of the lower part of glycolysis to oscillate [91]. This was based on the strong activation of pyruvate kinase by F16bP. Although forward activation does not so readily lead to oscillations as does backward activation (or forward inhibition), it can do so if the interaction is sufficiently nonlinear [124]. Since the concentration of F16bP during oscillations stays well above 1 mM, the activation of pyruvate kinase appears to be saturated (no further activation is observed above 0.5 mM F16bP [268]; chapter 8). This alternative mechanism of glycolytic oscillations may therefore not be very realistic. Another alternative to the ‘PFK-oscillophore theory’ is based on the autocatalytic stoichiometry of glycolysis. The surplus of ATP that is produced after investment of ATP at the first steps of glycolysis can be used to invest more ATP. Such a “turbo” principle (chapter 6) is another form of backward activation that has been shown to be able to introduce instability to the glycolytic system [79]. Models of glycolysis based on the autocatalytic stoichiometry of
glycolysis without taking the allosteric regulation of PFK into account, can also display oscillatory behavior [34, 79, 334].

**Intact cells versus cell-free extracts**

Most studies on glycolytic oscillations have been carried out in cell-free extracts. This is a very convenient system, but may not reflect the conditions that prevail in intact cells. Obviously, transport processes have been eliminated, some of which may be important ATP-consumers (such as the proton-translocating plasma membrane ATPase). Addition of an ATP-consuming enzyme, apyrase, led to an increase in the frequency [283], suggesting control of the ATP-consuming steps over the frequency (see next paragraph). This result may explain to some extent why yeast extracts with a similar protein content as that of the cytosol, exhibit much longer periods than intact cells (0.5-1 min for intact cells [284, 299, 300] compared to 5-15 min for cell-free extracts [80, 152, 277, 283, 284]).

There is another important and intriguing difference between extracts and populations of intact cells: in the latter system the oscillations in individual yeast cells should be synchronous for oscillations to be macroscopically observable. With $10^6$-$10^7$ cells ml$^{-1}$ in a cuvette, most cells must be largely in phase in order for the average NADH-signal to oscillate. The fact that the concentration of AMP oscillates with a relative amplitude above 90% of the average concentration [299], indicates that this synchronization is tight. Indeed, mixing of two populations that oscillate 180° out of phase, initially showed a diminished amplitude of the oscillation of the mixed population, but the oscillation recovered quickly to the original amplitude [116, 297]. The phase of the recovered oscillation was always in between the phases of the original oscillations [Bakker, Richard and Westerhoff, unpublished results]. Attempts to measure oscillations in single cells, using fluorescence microscopy, confirmed that under some conditions the average oscillation damped more quickly than that in individual cells [10, 68]. Damping of oscillations in populations of intact cells may be caused by (i) the relevant parameter values in each cell (all real parts of the eigenvalues of the system being negative, see section 1.2), (ii) by the ensemble of cells collectively setting a parameter value in time such that each cell's oscillation becomes damped after a while (such as the accumulation of ethanol [10]), or (iii) by ineffective synchronization of the cells. The reported growth-dependency of damping in suspensions of intact cells [300] may be related to any of these mechanisms, or a combination thereof. Part of chapter 3 will touch upon this problem.

The mechanism of synchronization has been a puzzle ever since such coupling of oscillations was observed [284, 404]. We have obtained strong evidence that acetaldehyde mediates the coupling [297]. Acetaldehyde induces phase shifts, which are both dose- and phase-dependent. The acetaldehyde concentration in the medium also oscillated, at the same frequency as that of the NADH oscillation. Analysis of free acetaldehyde in the medium was difficult, because acetaldehyde reacts with cyanide to form lactonitril [298, 413]. This trapping reaction
appears crucial for effective synchronization, as it keeps the free acetaldehyde concentration at a low level, where the relative amplitude in the concentration of acetaldehyde is sensed by alcohol dehydrogenase. The fact that a higher concentration of cyanide is required for optimal oscillations than needed for inhibition of respiration, confirms this hypothesis. Moreover, addition of too much cyanide leads to damped oscillations. The optimal concentration of cyanide varies with the conditions: e.g., galactose-grown cells require less cyanide than glucose grown cells [294].

Our experimental findings have been backed up by theoretical studies on the synchronization of glycolytic oscillations by Heinrich's group [405]. They found that a compound that rapidly diffuses into the medium can indeed lead to coupling, and thus synchronization, of oscillations. The coupling depends on the volume ratio of cells compared to the medium (i.e. the cell density, see [7, 10, 301]) and the rate of trapping of the coupling compound (i.e. the cyanide concentration). Interestingly, coupling between the cells could also lead to their desynchronization, forcing them to oscillate out of phase. This result may resolve the apparent contradiction between our view and the suggestion in the literature that acetaldehyde desynchronizes oscillations [116].

The amplitude of the acetaldehyde oscillation as reported by [297], however, was higher than we found when the experiments were repeated [Veldkamp and Teusink, unpublished results]. It appears that the actual concentration of acetaldehyde used for calibration by Richard et al. had been lower than was presumed [Richard, personal communication], probably due to the volatility of acetaldehyde. The standard used later did comply with Lambert-Beer's law and the extinction coefficient of NADH, and the concentration of acetaldehyde was found to oscillate between 15 and 30 μM (rather than 40-90 μM). These concentrations are lower than the threshold value for observing phase shifts with added acetaldehyde (which was about 30 μM [297]; the phase shifts were done with the correct standard). This appears to agree with results of the theoretical analysis. Preliminary calculations of phase shifts in the model of Wolf and Heinrich showed that a higher concentration of the coupling agent was required to obtain an observable phase shift simultaneously in all cells, than the concentration around which the coupling agent oscillates during synchronized oscillations [Teusink and Wolf, unpublished results].

Control of oscillatory characteristics: MCA of oscillations?

Although PFK may be the oscillophore of glycolysis, the extent to which the characteristics of the glycolytic oscillations are controlled by the activities of the (other) constitutive enzymes, remains an open question. If the oscillophore is the clock of the system that dictates it behavior to the other steps, control may be expected to reside in the oscillophore only. This is the main question of the first part of this thesis. In chapter 2 this question is
addressed by examination of the control properties of the enzymes in three different models of glycolytic oscillations. In chapter 5, experimental verification of the results of chapter 2 is presented. The reason to embark upon control analysis of oscillations is that limit-cycle oscillations can be considered the steady states of dynamic behavior. Studies on dynamic systems suffer from sensitivity to initial conditions (the integration constants $a$, $b$ and $c$ in Eq. 1.16). Those on limit-cycles, however, do not. One may therefore hope to be able to construct a control analysis for limit cycles similar to that for steady states (section 1.1). Considering the ubiquity of periodic phenomena in nature, such a control analysis should be extremely useful in the understanding of control and regulation of dynamic biological systems. Since amplitude and frequency of a limit cycle are time-independent, control coefficients for these variables can be easily defined [2, 389]:

$$C_i^\omega = \frac{\partial \ln \omega}{\partial \ln v_i}, \quad C_i^{A_k} = \frac{\partial \ln A_k}{\partial \ln v_i} \quad (1.19)$$

where $\omega$ is the angular velocity ($\omega = 2\pi v$ where $v$ is the frequency and $\omega=2\pi/\tau$ where $\tau$ is the period. All three quantities are expressions for the rate at which the system traverses the limit cycle) and $A_k$ is the amplitude of metabolite $k$. Summation theorems have also been derived [2, 389] (see also chapter 2).

For a full control analysis, however, connectivity theorems are required, which link the elasticities of the enzymes to the control coefficients. The eigenvalues are functions of unscaled elasticities (see section 1.2) and may therefore give a clue on how the control of frequency is connected to elasticities, at least for oscillations close to the Hopf bifurcation (where the first-order approach is valid). For realistic oscillations with large amplitudes, this is not an option. Moreover, the elasticities should be time-dependent (and periodic). A recent attempt to resort to time-dependent control coefficients shows that for autonomous limit-cycle oscillations such an approach cannot work [191]. The periodic control coefficient was defined as the (infinitesimal) difference in the time course of some metabolite concentration after an (infinitesimal) change in some enzyme activity $e_j$ [191]:

$$C_i^{X_j}(t) = \left[ \frac{X_j(t + T^*, e^*_i) - X_j(t + T, e_i)}{e^*_i - e_i} \right] \lim(e^*_i - e_i) \to 0 \quad (1.20)$$

It was emphasized that $X_j$ should be a periodic function of time and dependent on the concentration of the enzyme. The stars refer to the modified situation, i.e. $e^*_i$ is the modulated enzyme concentration, causing the period $T$ to change to $T'$. The problem is that the numerator of this control coefficient will increase in time, because of the difference in period caused by the
parameter change. The two oscillations will have an ever-increasing difference in phase. Even for infinitesimal modulation of enzyme activity, the numerator will become finite as time proceeds to infinity, making Eq. 1.20 undefined and therefore not useful [191]. A similar conclusion had been drawn by Larter et al. [215]. Thus, for autonomous limit-cycle oscillations, we can only resort to the analysis of the control of time-independent properties such as frequency and amplitude. The question is therefore whether frequency and amplitude control coefficients are zero for enzymes that are not the oscillophore (chapter 2 and 5).

The approach of periodic control coefficients does work, however, for forced oscillations, where the frequency is fixed by the external oscillator, and the control coefficient converges in time to a unique periodic function. The control of the enzymes over the frequency, however, will then obviously be zero. This approach may nevertheless be useful in studying, e.g., the control of the response to periodic changes of hormone levels in the bloodstream [124]. Also glycolytic oscillations may be studied in this way, if they are entrained by an external periodic supply of substrate. In such cases, however, complex dynamics may occur, such as period doubling and chaos [234].

The question, how to arrive at connectivity theorems for autonomous oscillatory systems, is still unanswered. Considering the finite amplitude of biologically relevant oscillations, time-dependent elasticities and control coefficients appear required. Perhaps a complete control analysis for autonomously oscillating systems is possible along the lines used for control analysis of organisms growing in chemostats. In such ‘flux-driven systems’, flux control coefficients can be determined via the effects on metabolite levels, even though the flux is completely controlled by the dilution rate [345]. Likewise, the response of enzymes to an external oscillatory input, in terms of the amplitude of (forced) oscillations in metabolite concentrations, might yield information about the control properties of these enzymes in the absence of the oscillating input.

1.4 Tools in the quantitative study of metabolism

1.4.1 Experimental approaches in MCA

For experimental control analysis, we require tools to modulate enzyme activities by some specific parameter change. The control coefficient can then be calculated if the response of the system to the parameter change can be measured and if the extent to which the enzyme activity is modulated by the parameter is known. The latter is not absolutely required if a full co-response analysis is possible. Here I will review a few approaches to modulate enzyme activities; more ways to calculate control coefficients have been described elsewhere [97, 98, 307].
The enzyme concentration: genetics

The most straightforward way to modulate enzyme activities is to modulate the amount of enzyme in the cell. Flint and coworkers used heterokaryons in *Neurospora crassa* to study the effect of different gene dosages on the arginine biosynthetic pathway [101]. Jensen and coworkers used an IPTG-inducible promoter, cloned in front of the (genomic) *atp* operon of *Escherichia coli*, to modulate the expression level of H⁺-ATPase to study its effect on growth rate [172, 173, 175]. Rohwer used the same strains to study the effect on the transport rate of methyl α-D-glucopyranoside (a glucose analogue). In *Salmonella typhimurium* and *E. coli* the control of the components of the glucose phosphotransferase system (PTS) on the rate of glucose uptake and on growth rate was determined by introduction of an IPTG-inducible promoter in front of the PTS genes on a plasmid [313, 371].

For yeast, regulatable promoters suitable for control analysis were not available until recently. The methionine-repressible promoter of *MET25* may be used [249], but it has the major disadvantage that methionine is metabolized, which obviously relieves the repression. Alternative promoters are now available. One is the copper-sensitive promoter, which has been claimed to be induced in a range of copper concentrations that is below the levels at which this metal ion show toxicity [229]. Another is the *tetO* promoter, which is sensitive to the antibiotic tetracycline [114]. Yet another promising system for controlled gene expression is based on the glucocorticoid-receptor mediated induction of gene expression, which is functional in yeast [321].

An alternative approach is the use of promoter deletions. The non-coding upstream region of many genes in yeast has regulatory sequences. Removal of these sequences may lead to altered expression levels. This approach is currently used in Karel van Dam's laboratory to modulate the expression level of hexose transporters *HXT1* and *HXT7* [Ye and Kruckeberg, personal communication]. Thus, important new tools have become available to specifically modulate the activity of enzymes in yeast by genetic means. A drawback of the genetic approach is that hierarchical effects may complicate the interpretation of the results in terms of traditional control analysis. This may also be considered an advantage, as it may lead to more insight in the homeostatic regulation of cell functioning. If the control analysis is performed with the aim of strain improvement via genetic modification, hierarchical effects are inevitable.

Titration with specific inhibitors

The activity of many enzymes can be decreased by the addition of a specific inhibitor. The inhibition approach has e.g. been used for oxidative phosphorylation, where many specific inhibitors are known [134, 138]. Buttgereit used different inhibitors for ATP-consuming processes [57]. The inhibitor phloretin was used in the study of the control of glucose transport on the glycolytic flux of *Trypanosoma brucei* [13]. A similar method will be described in chapter 5 for glucose transport in yeast, with maltose as the inhibitor.
One problem with the inhibitor-titration approach can be the quantification of the extent to which the enzyme is inhibited. Especially the use of competitive inhibitors is complicated, as their impact also depends on the relevant metabolite concentrations. As both phloretin and maltose inhibit transport competitively with respect to glucose, complications arise concerning the elasticity of the transporter to the inhibitor. These complications have been analyzed by Bakker [13].

1.4.2 Problems associated with the measurement of fluxes and metabolites

Control analysis requires accurate measurement of metabolic variables. The measurement of fluxes is relatively simple by monitoring the concentration of the extracellular substrate and/or the products in time. Frequently used methods are enzymatic analyses high-performance liquid chromatography (HPLC), and on-line mass spectrometry of gases. A method that will be employed in this thesis that was not previously used for glycolytic flux measurements, is microcalorimetry [137, 347]. Although microcalorimetry does not measure a specific flux, it measures the rate of heat liberation of a system, which can be related to chemical reactions taking place, as the heat released by a system under isobaric conditions is equal to the enthalpy change of the system. This follows directly from the first law of thermodynamics and the definition of enthalpy [137, 396]. Heat flux measurements are extremely sensitive, and in that respect superior to indirect flux measurements via the concentrations of substrates and products. It can therefore be a valuable addition to the latter technique (see chapters 3 and 5).

Measurement of the concentration of intracellular metabolites is much more complicated. Whenever metabolites are to be measured accurately, there are four important conditions to be met. First, metabolism should be quenched as fast as possible and metabolic processes should be inactive during subsequent treatment of the samples. Second, the extraction of metabolites should be quantitatively complete (protein-bound metabolites being a serious complication). Third, the total amount of metabolites should be sufficiently large to permit the subsequent analyses to be performed accurately. Fourth, if the metabolite also occurs in the extracellular medium (such as pyruvate and glycerol), cells must be separated from that medium before the metabolites are extracted.

Several methods to extract metabolites from yeast have been advocated in the literature. The most frequently used method is perchloric acid or trichloroacetic acid extraction [136, 314, 360, 386], sometimes supplemented with the detergent digitonin [381]. Extraction and quenching of metabolism occur simultaneously. No separation between intracellular and extracellular metabolites is possible. The method is quick and works well for a large number of acid-stable compounds that are present in the cell in the millimolar range. For acid-labile compounds or compounds that are present in very small quantities, other methods have been developed. De Koning et al used cold methanol at −40 °C to quench metabolism. Because the...
cells were not permeabilized by the methanol, they were able to concentrate the sample by centrifugation [82] (chapter 4). Chloroform was used to denature the enzymes and to extract the metabolites. The method is performed at neutral pH and in principle allows extraction of any metabolite with a favorable partition coefficient for water over chloroform. An alternative method was developed by the group of Francois, which employs boiling in buffered ethanol [128]. This can be done directly, by adding sample to boiling ethanol, or by quenching in methanol first and after centrifugation, adding boiling ethanol to the cell pellet. This method also works at neutral pH and is simpler than the chloroform extraction, but it requires removal of the ethanol by evaporation or lyophilisation prior to the actual metabolite analysis.

Although each method gives reproducible results with standard deviations of about 10% or less, substantial differences may be obtained with the different extraction methods. These differences are dependent on the nature of the particular metabolite and are poorly understood. Two examples are given in Table I. Experiment I compares ethanol boiling with PCA extraction; experiment II compares PCA extraction with and without prior quenching in cold methanol (experiments I and II should not be compared).

As can be seen from Table I, absolute metabolite concentrations have a certain degree of uncertainty, which varies between metabolites. In experiment II, the lower concentration of pyruvate after methanol quenching is caused by the contribution of extracellular pyruvate in the direct PCA extraction. The lower concentration of fructose 6-phosphate after methanol quenching, however, cannot be caused by extracellular fructose 6-phosphate. This lower concentration of F6P was also seen for ethanol extraction, but does not seem to be caused by residual activity of phosphoglucone isomerase activity at -40 °C, as a pgiA mutant showed the same drop in fructose 6-phosphate when cells were quenched in methanol [Esgalhado and Teusink, unpublished results]. Smits found that the ATP level measured after quenching in methanol and extraction with chloroform, was only 50% of the ATP level found after direct quenching and extraction in PCA [342]. Since only 25% of the ATP was lost by quenching in methanol, the extraction by chloroform may not be as complete as compared to extraction with PCA. For most metabolites, ethanol extraction appears slightly more efficient than PCA extraction, although not for NAD.

These results illustrate the difficulty of obtaining absolute concentrations of intracellular metabolites. Other complications include the uncertainty of the free concentration of metabolites (some may be bound to enzymes or other structures in the cell) and compartmentation of metabolite pools. These problems may be less dramatic for control analysis, where in many cases it would suffice if the extraction were proportional to the internal concentration (and as long as the same extraction procedure is used). It is a problem for other applications e.g., model validation, where absolute values are required.
### Table I. Comparison of different metabolite extraction techniques. Numbers are expressed in nmol extracted from 0.5 ml of sample ± standard deviation of the mean of 4 determinations. PCA: perchloric acid extraction; EtOH: buffered ethanol extraction according to [128]; MeOH quench: samples were quenched in cold methanol, centrifuged and the pellets were extracted with PCA.

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>G6P (nmol)</th>
<th>F6P (nmol)</th>
<th>ATP (nmol)</th>
<th>PYR (nmol)</th>
<th>NAD (nmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>PCA</strong></td>
<td>20.7 ± 1.0</td>
<td>4.2 ± 0.1</td>
<td>25.2 ± 1.6</td>
<td>92.3 ± 5.3</td>
<td>5.6 ± 0.2</td>
</tr>
<tr>
<td><strong>EtOH</strong></td>
<td>32.1 ± 1.9</td>
<td>6.9 ± 0.3</td>
<td>26.3 ± 1.5</td>
<td>115.1 ± 1.8</td>
<td>3.9 ± 0.1</td>
</tr>
<tr>
<td><strong>Experiment I</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>PCA</strong></td>
<td>29.7 ± 2.0</td>
<td>8.9 ± 0.2</td>
<td>39.6 ± 0.5</td>
<td>26.6 ± 1.0</td>
<td>5.0 ± 0.2</td>
</tr>
<tr>
<td><strong>direct</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>PCA</strong></td>
<td>34.1 ± 1.3</td>
<td>4.9 ± 0.2</td>
<td>30.9 ± 0.3</td>
<td>13.1 ± 0.3</td>
<td>4.2 ± 0.1</td>
</tr>
<tr>
<td><strong>MeOH quench</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1.4.3 Mathematical modeling

An important tool in quantitative biochemistry of metabolism is mathematical modeling [143, 147]. This thesis makes extensive use of that technique. Modeling can serve different purposes. It may be used to identify the key structures in metabolism that may give rise to a certain qualitative behavior. Models constructed with this purpose are often called core models and have only limited kinetic detail. They aim not so much at quantitative but rather at qualitative correspondence between model and experiment. Core models are used in chapter 2 to study whether control of frequency and amplitude is in principle distributed among different enzymes or not. In chapters 6 and 7 core models of glycolysis are used to relate the stoichiometry of glycolysis to the need for feedback regulation of hexokinase.
More elaborate models may serve to make quantitative predictions of metabolic behavior. For example, they may be used to calculate the control coefficients of the enzymes of the pathway, or to examine the impact of large changes in parameters. This type of modeling has been pioneered by Garfinkel and colleagues [113]. Quantitative agreement between model and experiment is required for this approach to be useful and therefore model validation is very important in this type of modeling (see chapter 8). Realistic modeling has been used for glycolysis of erythrocytes, to predict the pathological consequences of enzyme deficiencies in humans [288, 330]. The glucose phosphotransferase system in E. coli has been modeled in detail and showed quantitative agreement between model and experimental results. The predicted control coefficients agreed well with the measured ones, and the model offered an explanation for discrepancies between protein-dependency of the in vivo and in vitro uptake rates [307, 308]. Glycolysis of Trypanosoma brucei has been modeled, with the aim to increase drug selectivity, by identifying those steps in glycolysis whose inhibition would affect the glycolytic rate to the largest extent [13, 14]. The TCA-cycle has been modeled in Dictyostelium discoideum [407, 408]. Large differences were found between in vitro kinetics and in vivo activities of enzymes involved in that pathway. Also for yeast glycolysis detailed models exist [77, 78, 108, 303, 305, 324]. In chapter 8 we present our version of a detailed model of yeast glycolysis and we discuss differences in strategy between our and existing models.

Another approach to modeling is so-called flux analysis (see many examples in a recent special issue of Biotech. Bioeng. (1998) Vol. 58, No. 2-3). These models are based on steady state mass-balances and stoichiometric constraints. They allow the calculation of fluxes through the metabolic map on the basis of input and output variables (see for an example [259]). Such models may indicate potential bottlenecks in metabolism. Flux analysis does not make use of kinetic information regarding the constituent enzymes. Attempts are being made, however, to merge flux analysis with kinetic modeling [Visser, Van der Heijden, Heijnen, personal communication]. Flux analysis can be useful for kinetic modeling, e.g. to calculate the fluxes through branch reactions that should be described by the model [305] (see for a discussion of different methods in metabolic engineering, including flux analysis, control analysis and modeling, [258]).

1.5 Outline of this thesis

This thesis deals with the understanding of the systemic behavior of glycolysis of Saccharomyces cerevisiae on the basis of the properties of the constituent enzymes and their interactions.

In chapter 2 the question is addressed whether control of dynamic states, in this case limit-cycle oscillations, can be distributed over the enzymes of the pathway, or whether all
control must reside in the enzyme held responsible for the generation of the oscillations: the oscillophore. For three core models of glycolysis it was found that control of dynamic states does not differ from steady state control, in that control was shared by all enzymes of the pathway. The summation theorems for frequency and amplitude control coefficients, previously derived theoretically, were confirmed. Chapter 3 reports on how the dynamics of glycolysis in intact cells have been studied by microcalorimetry. This was quite a technical challenge, considering the short period of the oscillations and the large response time of calorimeters. It was investigated to what extent the measurement of heat flux may yield additional information about the oscillatory system.

The potential of microcalorimetry as a sensitive technique to measure fluxes was identified in chapter 3 and subsequently used in chapter 5 for control analysis of the average flux during the oscillations. Differences in specific heat flux were found between cells harvested at different points during growth on glucose, which pointed to altered metabolic fluxes. These were identified and related to the different dynamic behavior of the cells at different growth phases [300]. In chapter 5 attempts are described to measure frequency and flux control coefficients of the hexose transporter during glycolytic oscillations.

Measurement and understanding of the control of the hexose transporter depend on the way glucose transport is regulated by internal signals. The analysis of chapter 5 is therefore preceded by a study, in chapter 4, on the regulation of the transporter. Conflicting results can be found in the literature. We demonstrate that for high affinity glucose transport, the simplest model of facilitated diffusion suffices to explain a 50% reduction in the steady-state transport rate compared to the zero trans-influx rate. This conclusion is based on the measurement of the intracellular glucose concentration in glucose-fermenting yeast cells, for which we have developed a new assay. For low-affinity transport, however, we present evidence that the carrier must become more active in time, because the rate of zero trans-influx of glucose was lower than the steady-state glucose consumption rate. This activation may result in an underestimation of the zero trans influx kinetics, measured during the first 5s after glucose addition.

Various ways of modulating the rate of hexose transport were used in chapter 5 to measure control exerted by the hexose transporter. The usefulness of each approach is discussed. It is concluded that the extent to which the glucose transporter controls the average flux during glycolytic oscillations varies with the external glucose concentration. At high glucose concentrations (far above the $K_m$ of the transporter for glucose), the control is much lower than at low glucose concentrations, where the flux control coefficient eventually reached 1. For the control of the frequency, it was shown that the control exerted by the hexose transporter is significant but lower than 1. This demonstrates that the oscillophore PFK does not solely control the frequency and that control of frequency is distributed over at least two enzymes.
Having improved our understanding of the control and regulation of the hexose carrier, we moved to the next step in glycolysis: hexokinase. As outlined in section 1.3.1, hexokinase is regulated by Tps1. In chapter 6 we have constructed a core model of glycolysis to demonstrate how the stoichiometry of glycolysis demands regulation of hexokinase, other than via the adenine nucleotides alone. The core model was able to describe and explain the phenotype of the \( \text{tps1}^{-} \) mutant, as well as that of a suppressor of the \( \text{tps1}^{-} \) phenotype.

Chapter 7 extends the analysis by applying control analysis to the core model of chapter 6. The analysis highlights the importance of the stoichiometry of metabolic pathways in relation to the requirement of feedback sensitivity of enzymes. The analysis also leads to the identification of “slave enzymes”, which are enzymes that cannot control any variable of the system except one. The lack of control is propagated downstream the reaction sequence, as long as these downstream reactions form a monofunctional unit with the slave enzyme. This analysis emphasizes the importance of product sensitivity for the control distribution of a metabolic system. This effect has not received the attention it deserves in many existing models of yeast glycolysis, as model simplifications frequently include the use of irreversible reaction kinetics (see discussion of chapter 8).

In chapter 8 the knowledge about the regulation of hexose transport and hexokinase have been used in a detailed kinetic model of glycolysis. For this purpose, a large set of kinetic parameters of the glycolytic enzymes have been measured in a single industrial strain, for a single set of experimental conditions. In vivo fluxes and metabolite concentrations have been determined to validate the model. Despite the compatibility of the data sets, the in vitro kinetics of a large set of enzymes are unable to describe the in vivo behavior. A close inspection of the differences identified the enzymes most poorly describing the in vivo function. The model therefore served as an important heuristic tool in showing the lack of biochemical information that is still present after 100 years of biochemistry on yeast glycolysis.

Finally, in chapter 9 the results described in this thesis are put in perspective and I give my view on some key regulatory features of the glycolytic pathway, for which I suggest future work to prove me wrong.