Exposing a complex metabolic system: glycolysis in Saccharomyces cerevisiae
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8.1 Summary

In this chapter it was attempted to describe the in vivo behavior of yeast glycolysis in terms of the in vitro kinetic properties of the constituent enzymes. To that purpose, a realistic model of glycolysis in resting, anaerobic compressed yeast was constructed. Under these conditions fluxes and metabolite levels were measured. In the same yeast, a set of kinetic parameters was determined for most glycolytic enzymes. This set was supplemented with literature values.

In a first model, branch reactions were ignored. This model failed to reach a stable steady state. Introduction of branches towards trehalose, glycogen, glycerol and succinate did allow such a steady state. The model predictions were compared with the experimentally determined system behavior. For each enzyme it was calculated how much the in vitro rate differed from the in vivo flux. Half of the enzymes predicted the in vivo behavior within a factor of 2. Possible explanations for larger discrepancies for the other enzymes are discussed. Other difficulties are outlined and experimental strategies are suggested for model improvement.

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1 In collaboration with Jutta Passarge, Jacky L. Snoep, Corinne A. Reijenga, Eugenia Esgalhado, Coen C. van der Weijden, Mike Schepper, Michael C. Walsh, Karel van Dam and Hans V. Westerhoff
8.2 Introduction

In the post-genome era, for an increasing number of organisms, all the genetic components that suffice to constitute a living cell are known. The elucidation of the molecular function of the proteins encoded by the many novel genes is a major challenge in biology today [264]. Accordingly, the ambition is to understand functioning in and of the living cell from the properties of these components. This may be done by detailed mathematical modeling, using the enzyme-kinetic properties determined in vitro. However, the tenet that the behavior of the living cell or of the functioning of enzymes in that living cell can be calculated from the kinetic properties determined in vitro, is not undisputed. The conditions in the living cell can differ drastically from those in a test tube. For one, enzymes are often characterized under conditions that are optimal for that enzyme, e.g., in terms of temperature and pH. These may differ between the enzymes in a single cellular compartment and be again different from the actual conditions in the living cell. Regulation of the activity of enzymes by metabolites produced elsewhere in cell metabolism may be overlooked. Moreover, the inside of the cell is compartmented and enzymes that are present in a classically defined compartment may be subcompartmented due to binding to membranes, the cytoskeleton or to other enzymes. In the latter case, metabolites may be channeled between enzymes rather than that the enzymes are confronted with substrates and products at thermodynamically well defined concentrations [348]. In addition, the concentration of enzymes in the cell is much higher than in the usual test tube experiment, a phenomenon that may be greatly enhanced by macromolecular crowding [193, 348]. Perhaps finally, there is the possibility that the functioning of living cells depends extremely critically on the magnitude of its kinetic parameters, e.g. if cells were to operate near bifurcation points. In that case the functional behavior of cells could be incalculable in principle. Although in many of these considerations there appears to be some truth, it remains to be seen whether they indeed compromise the ability to understand the functional behavior of the living cell. This paper is aimed at examining this issue.

It should be noted that thereby the aim of this paper differs drastically from that of most modeling papers. Many of the already existing mathematical models of metabolic or other pathways are meant to examine what type of dynamic behavior such a pathway might exhibit at any values of its kinetic parameters. In this way it has been shown that there are magnitudes of the parameters characterizing the kinetic and regulatory properties of the glycolytic enzymes that lead to oscillations [40, 232]. Because they only aim at showing that certain kinds of behavior are possible in principle, these models do not aim at incorporating the genuine, experimentally determined kinetic properties of the enzymes. Often they are even simplified to so-called 'core models' which leave out or combine reactions that are not considered relevant for
the phenomenon of interest (see also chapter 2, 6 and 7). Virtually all the other existing models aim at describing the functional behavior of the metabolic pathway, without the requirement that the genuine kinetics of the enzymes are used in the kinetic equations. In some cases phenomenological equations are used for the pathway as a whole, in other cases such equations are used for the enzymes thereby omitting reference to the actual biochemical mechanisms. Or, even, if the models start out with the genuine kinetic equations, rate constants are fitted so as to obtain a model that fits the behavior of the pathway, even though the kinetics of the enzymes are then inconsistent with their in vitro kinetics.

Most of these models are well suited for their specific purpose, but they are not useful for the purpose of testing whether we can understand pathway behavior from the genuine kinetic behavior of the enzymes. For this particular aim, it is necessary (i) to employ experimentally determined enzyme kinetics; (ii) to refrain from adjusting kinetic parameters to obtain best fits to pathway behavior, and (iii) only to make model simplifications after extensive demonstration that the simplifications are quantitatively without effect. The realistic detailed kinetics type of model we are here requiring, has been pioneered by Garfinkel and coworkers [113]. However, that early work was much too optimistic. It did not even question if such a model would describe cell function, but just assumed it would. This early initiative got bogged down in the problem that to predict behavior of the systems that were modeled (large parts of cell metabolism), the values of many kinetic parameters needed to be known, whereas at that time and even now (see below) the number of kinetic parameters that is known sufficiently accurately under the relevant conditions is very small. Wright and colleagues have since engaged in a major quantitative analysis of sugar and tricarboxylic acid metabolism in Dictyostelium discoideum, indeed combining in vitro biochemistry with measurements in the intact cell [408]. They did not reach a correspondence between what they calculated on the basis of the in vitro kinetics and what they measured in the intact cell. Also in this case however, the metabolic pathway analyzed was fairly complex and there were uncertainties regarding the efficiency of extracting metabolites and enzymes from the cells prior to their quantitative characterization. More recently, Rizzi et al. constructed a detailed kinetic model of yeast metabolism [305]. Their primary interest was to describe their experimental observations of glycolytic transients in growing cells [360]; for this extensive system, they had fitted their $V_{\text{max}}$’s in accordance with the flux, thereby relaxing our condition (ii) from above.

Reading about these early experiences made us tune down our ambitions. To examine if in vivo behavior may (already) be calculable from the known kinetic properties, we decided to examine the most promising case, i.e., that of a metabolic pathway with a major flux, in an organism in which the enzymes are present at high concentrations, and in a single compartment. Moreover it should be possible to determine the metabolite concentrations and the flux reliably, and the enzymes of the pathway should already have been characterized most extensively. Yeast glycolysis is one of the few pathways meeting these criteria.

Mathematical model of yeast glycolysis
Glycolysis in *Saccharomyces cerevisiae* has been studied extensively for more than a century now. All the components that constitute the pathway appear to have been identified and characterized both biochemically and kinetically, and one may conjecture that this pathway stands the highest chance of having the important metabolic regulatory interactions identified. The concentrations of the enzymes are so high that diffusion should be competent to have metabolites sample many enzyme molecules at the time scale of metabolic turnover [398], making pool behavior likely. Furthermore, with the functional analysis of the yeast genome being highly active, the possibilities of further progress if the kinetic calculations would fail to describe function, would seem high.

In this study we collect a data set of in vitro kinetics of all glycolytic enzymes, largely by measuring them under one standard condition. In the same cells we have gathered data on the overall behavior of the glycolytic system, i.e. fluxes and metabolite levels. We have used conditions of anaerobic glucose fermentation without growth to study the behavior of the glycolytic pathway. This system is of sufficient complexity to test our knowledge of yeast glycolysis, and avoids the introduction of many unknowns associated with (respiratory) growth. We employ mathematical modeling to test whether the best available knowledge of the kinetic properties of the glycolytic enzymes allows us to understand the overall behavior of the pathway. We report that for about half of the enzymes it does not and we show what the differences could be between the in vivo enzyme properties and our in vitro knowledge of them.

### 8.3 Methods and Materials

**Stoichiometry and moiety conservations**

The model includes most enzymes of the metabolic pathway from glucose uptake to alcohol dehydrogenase (Fig. 1). The reaction catalyzed by TPI was very close to equilibrium when explicit kinetics were used in the model (the mass-action ratio divided by the equilibrium constant was $>0.95$). AK should be in equilibrium when the system is at steady state, and the experimentally determined levels of adenine nucleotides confirm this within experimental error (see Table III). Accordingly, triosephosphate isomerase and adenylate kinase were taken to be in equilibrium; all the other enzymes were included with explicit enzyme kinetics. In the boxes the branches are indicated that were introduced in the branched version of the model. From the reaction stoichiometries of the unbranched glycolytic pathway, three moiety conservations [163] were derived. The first corresponds to the conservation of the adenine nucleotides, the second...
to conservation of the nicotinamide nucleotide moiety, and the third to the conservation of redox equivalents (see also [288]):

\[
\begin{align*}
[ATP] + [ADP] + [AMP] &= \Sigma_1 \\
[NAD] + [NADH] &= \Sigma_2 \\
[BPG] + [3PGA] + [2PGA] + [PEP] + [PYR] + [AcAld] + [NAD] &= \Sigma_3
\end{align*}
\] (8.1) (8.2) (8.3)
The latter conservation does not occur in the branched glycolysis model: there the pool size \( \Sigma \) can vary (see results and discussion of the branched model). The conservation sums \( \Sigma \) are parameters of the model the magnitude of which can be estimated by the measured metabolite and cofactor concentrations (see Table I).

**Rate equations**

In this section, the kinetic equations used in the model are described. Experimental kinetic data, assumptions and choices for particular data sets in the literature will be motivated and discussed in Appendix I; the kinetic parameters are found in Table II.

The transport of glucose across the cell membrane occurs via facilitated diffusion [106, 213, 343], which is described by a symmetrical carrier model:

\[
\frac{[\text{Glc}_{\text{out}}] - [\text{Glc}_{\text{in}}]}{v_{\text{transport}}} = \frac{\text{K}_{\text{Glc}}}{1 + \frac{[\text{Glc}_{\text{out}}] + [\text{Glc}_{\text{in}}]}{\text{K}_{\text{Glc}}}} \times \frac{[\text{Glc}_{\text{out}}]}{[\text{Glc}_{\text{in}}]}
\]

(8.4)

in which \([\text{Glc}_{\text{out}}]\) and \([\text{Glc}_{\text{in}}]\) are the concentrations of extracellular and intracellular glucose, respectively. The "interactive constant" \(K_i\) depends on the relative mobility of the unbound and bound carrier [199, 200] (chapter 4).

Irreversible Hill kinetics were used for PDC:

\[
v_{\text{PDC}} = v^+ = \frac{\left(\frac{[\text{PYR}]}{\text{K}_{0.5}}\right)^n}{1 + \left(\frac{[\text{PYR}]}{\text{K}_{0.5}}\right)^n}
\]

(8.5)

One substrate, one product reversible Michaelis-Menten kinetics was used to describe the enzymes PGI, PGM and ENO:

\[
v = v^+ = \frac{\frac{a}{\text{K}_a} \left(1 - \frac{\Gamma}{\text{K}_{eq}}\right)}{1 + \frac{a}{\text{K}_a} + \frac{p}{\text{K}_p}}
\]

(8.6)
where $a$ and $p$ represent the concentrations of the corresponding substrate and product, respectively. $\Gamma$ is the mass-action ratio, $p/a$, $K_{eq}$ is the equilibrium constant, $p_{eq}/a_{eq}$. Reversible Michaelis-Menten kinetics for two non-competing substrate-product couples was used for HK, GAPDH, PGK and PYK:

$$v = V^+ \frac{ab}{K_a K_b \left(1 - \frac{\Gamma}{K_{eq}} \right)} \left(1 + \frac{a}{K_a} + \frac{p}{K_p} \right) \left(1 + \frac{b}{K_b} + \frac{q}{K_q} \right)$$

(8.7)

where $a$ and $b$ represent the concentrations of the substrates and $p$ and $q$ the concentrations of the products.

Aldolase has been described by an ordered uni-bi reaction in which GAP dissociates first [63, 303]. The equation is:

$$v_{ALD} = V^+ \frac{a}{K_a \left(1 - \frac{\Gamma}{K_{eq}} \right)} \left(1 + \frac{a}{K_a} + \frac{p}{K_p} + \frac{q}{K_q} + \frac{aq}{K_a K_{iq}} + \frac{pq}{K_p K_q} \right)$$

(8.8)

where $a$ represents [F16bP], $p$ represents [DHAP] and $q$ represents [GAP].

ADH follows ordered bi-bi kinetics, with the cofactor binding first [112]:

$$v_{ADH} = \frac{V^+ \frac{ab}{K_{ia} K_b} - \frac{pq}{K_p K_{iq}}}{1 + \frac{a}{K_{ia}} + \frac{b}{K_{ia} K_b} + \frac{K_q b}{K_{ia} K_{iq}} + \frac{q}{K_{iq}} + \frac{ab}{K_{ia} K_b} + \frac{K_{aq} p}{K_{ia} K_{iq} K_p}} + \frac{K_{aq} b q}{K_{ia} K_{b} K_{iq}} + \frac{pq}{K_{p} K_{iq}} + \frac{ab p}{K_{ia} K_{b} K_{ip}} + \frac{b p q}{K_{ia} K_{b} K_{iq}}$$

(8.9)

where $a$ is [ethanol], $b$ is [NAD], $p$ is [acetaldehyde] and $q$ is [NADH].

Phosphofructokinase was described by a two substrate Monod, Wyman, Changeux model for allosteric enzymes [245] as developed by Hess et al [155] and used in, e.g. [108], with F6P and ATP as substrates and ATP, AMP, and F26bP as allosteric effectors. F16bP sensitivity was modeled via its negative effect on F26bP stimulation [268]. The rate equation and further details of PFK can be found in Appendix II.
For glycolysis to proceed, the net ATP produced by PGK and PYK should be consumed by ATP consuming processes. These processes are lumped into one general ATPase, whose activity is set to be dependent on ATP, according to experimental observations [57]. A linear relation was used:

\[ v_{\text{ATPase}} = k_{\text{ATPase}} \cdot [\text{ATP}] \]  

\[ (8.10) \]

**Differential equations**

For the two enzymes that were considered in equilibrium, the reactants were treated as one metabolic pool. The following metabolic pools were defined:

\[ [\text{Trio-P}] = [\text{DHAP}] + [\text{GAP}] \]  

\[ (8.11) \]

\[ P = 2[\text{ATP}] + [\text{ADP}] \]  

\[ (8.12) \]

The variable P has the physical meaning of the sum of high-energy phosphates (see also [14]). Due to the conservation of adenine nucleotides and the equilibrium constraint on AK, the adenine nucleotides are not independent of each other, but can be described by only one free variable. The variable P was chosen as the independent variable to describe the involvement of high-energy phosphate in the reactions. It is related to the energy charge and the phosphorylation potential [396].

A set of ordinary differential equations was used to describe the time-dependence of the metabolite concentrations:

\[ \frac{d[\text{Glc}]}{dt} = v_{\text{transport}} - v_{\text{HK}} \]  

\[ (8.13) \]

\[ \frac{d[\text{G6P}]}{dt} = v_{\text{HK}} - v_{\text{PGI}} - 0.5v_{\text{trehalose}} - v_{\text{glycogen}} \]  

\[ (8.14) \]

\[ \frac{d[\text{F6P}]}{dt} = v_{\text{PGI}} - v_{\text{PFK}} \]  

\[ (8.15) \]

\[ \frac{d[\text{F16bP}]}{dt} = v_{\text{PFK}} - v_{\text{ALD}} \]  

\[ (8.16) \]

\[ \frac{d[\text{Trio-P}]}{dt} = 2v_{\text{ALD}} - v_{\text{GAPDH}} (-v_{\text{glycerol}}) \]  

\[ (8.17) \]

\[ \frac{d[\text{BPG}]}{dt} = v_{\text{GAPDH}} - v_{\text{PGK}} \]  

\[ (8.18) \]

\[ \frac{d[\text{3PGA}]}{dt} = v_{\text{PGK}} - v_{\text{PGM}} \]  

\[ (8.19) \]

\[ \frac{d[\text{2PGA}]}{dt} = v_{\text{PGM}} - v_{\text{ENO}} \]  

\[ (8.20) \]

\[ \frac{d[\text{PEP}]}{dt} = v_{\text{ENO}} - v_{\text{PYK}} \]  

\[ (8.21) \]

\[ \frac{d[\text{PYR}]}{dt} = v_{\text{PYK}} - v_{\text{PDC}} \]  

\[ (8.22) \]

\[ \frac{d[\text{AcAld}]}{dt} = v_{\text{PDC}} - v_{\text{ADH}} (-2v_{\text{succinate}}) \]  

\[ (8.23) \]

\[ \frac{d[P]}{dt} = -v_{\text{HK}} - v_{\text{PK}} + v_{\text{PGK}} + v_{\text{PYK}} - v_{\text{ATPase}} (-v_{\text{trehalose}} - v_{\text{glycogen}}) \]  

\[ (8.24) \]

\[ \frac{d[\text{NADH}]}{dt} = v_{\text{GAPDH}} - v_{\text{ADH}} (-v_{\text{glycerol}} + 3v_{\text{succinate}}) \]  

\[ (8.25) \]

\[ \frac{d[\text{NAD}]}{dt} = -v_{\text{GAPDH}} + v_{\text{ADH}} (+v_{\text{glycerol}} - 3v_{\text{succinate}}) \]  

\[ (8.26) \]
The branch reactions are put in brackets. The concentrations of the metabolites in the equilibrium pools were calculated from the pool size and the corresponding equilibrium equations. For TPI:

\[ K_{eq,TPI} = \frac{[\text{GAP}]}{[\text{DHAP}]} \quad (8.27) \]

Combining Eq. 8.27 with Eq. 8.11 gives:

\[ [\text{DHAP}] = \frac{[\text{TrioP}]}{1 + K_{eq,TPI}} \quad (8.28a,b) \]
\[ [\text{GAP}] = \frac{[\text{TrioP}] \cdot K_{eq,TPI}}{1 + K_{eq,TPI}} \]

For the adenine nucleotides the equilibrium equation is as follows:

\[ K_{eq,AK} = \frac{[\text{AMP}] [\text{ATP}]}{[\text{ADP}]^2} \quad (8.29) \]

Solving the three equations Eq. 8.1, 8.12 and 8.29 for the three unknowns gives for ATP:

\[ [\text{ATP}] = \frac{-b + \sqrt{b^2 - 4ac}}{2a} \]

with

\[ a = 1 - 4K_{eq,AK} \]
\[ b = \Sigma_1 - P(1 - 4K_{eq,AK}) \]
\[ c = -K_{eq,AK} P^2 \]

The concentrations of ADP and AMP follow from equations 8.12 and 8.1, respectively.

The branched model included the following additional reactions:

2 G6P + P \rightarrow \text{trehalose} \quad (8.31)
G6P + P \rightarrow \text{glycogen} \quad (8.32)
DHAP + NADH \rightarrow \text{glycerol} + \text{NAD} \quad (8.33)
2 AcAld + 3 NAD \rightarrow \text{succinate} + 3 \text{NADH} \quad (8.34)
The fluxes to trehalose and glycogen were introduced as constants at the experimentally
determined values. For glycerol metabolism, it was assumed that glycerol 3-phosphate
dehydrogenase completely controlled the flux through that pathway, as experimental evidence
has shown that its control should be high [243, 255, 416]. Reversible Michaelis-Menten kinetics
(Eq. 8.7) was used with DHAP and NADH as substrates and glycerol 3-phosphate and NAD as
products. The glycerol 3-phosphate concentration was fixed at the measured value of 0.15 mM
(see Table I).

The formation of glycerol leads to a redox imbalance in glycolysis, as NADH is oxidized in
the process [372]. We have measured production of pyruvate, acetate and succinate. These weak
carboxylic acids can account for 95% of the glycerol formation, with the production of succinate
accounting for 80% of the glycerol production. For simplicity, we have only included a branch to
succinate to counterbalance the glycerol branch. This branch from acetaldehyde towards
succinate via the glyoxylate cycle comprises many steps and was necessarily simplified by:

\[ v_{\text{succinate}} = k_{\text{succinate}} \cdot \text{[AcAld]} \] (8.35)

The differential equations were integrated on a personal computer using the metabolic
modeling software SCAMP [315].

Measurement of enzyme kinetics
Compressed yeast Koningsgist from DSM Bakery Ingredients was used in all experiments.
Glucose transport was measured for intact cells resuspended in 100 mM KH₂PO₄ buffer, pH 6.5,
as described by [383]. To measure the kinetics of intracellular enzymes (except for PFK and PK),
1 g of yeast was resuspended in 1 ml ice-cold Millipore water. The suspension was diluted 20
times with 20 mM KH₂PO₄, pH 7.0, containing 1 mM of the protease inhibitor phenyl methyl
sulphonyl fluoride (PMSF). Acid-washed glass beads (1 g, 0.4-0.5 mm diameter) were added to 1
ml of cell suspension in an Eppendorf tube, and the mixture was shaken for 15 min at 4°C. The
extract was centrifuged (Eppendorf centrifuge, 15 min, maximal speed) at 4°C and the
supernatant was stored on ice until further use. The extract was diluted in 20 mM KH₂PO₄, pH
7.0. All enzyme assays were carried out in 50 mM PIPES buffer of pH 7.0 containing 100 mM KCl
and 5 mM MgSO₄. A COBAS BIO (Roche, Basel) automated analyzer was used for spectroscopic
measurement of the metabolite concentrations through NAD(P)H-linked assays.

Hexokinase was measured with 0.2 mM NADP and 0.56 U glucose 6-phosphate
dehydrogenase and ATP and glucose at varying concentrations.

Phosphoglucoisomerase was measured in the forward direction in the presence of 0.15
mM NADH, 1 mM ATP, 2.5 U phosphofructokinase, 0.3 U aldolase, 10 U triosephosphate
isomerase and 0.85 U glycerol 3-phosphate dehydrogenase and glucose 6-phosphate as
substrate. For the reverse reaction, 0.2 mM NADP and 0.56 U glucose 6-phosphate dehydrogenase was used with fructose 6-phosphate as substrate.

Aldolase was assayed with 0.15 mM NADH, 10 U triosephosphate isomerase and 0.85 U glycerol 3-phosphate dehydrogenase and fructose 1,6-bisphosphate as a substrate.

Glyceraldehyde 3-phosphate dehydrogenase was measured in the forward direction with 2 mM NAD, 10 mM KH$_2$PO$_4$, 0.9 mM EDTA, 0.2 mM dithioerythritol (DTT), 10 U triosephosphate isomerase and 18 mM DHAP as a substrate. For the reverse reaction, the assay contained 0.15 mM NADH, 1 mM ATP, 0.9 mM EDTA, 0.2 mM dithioerythritol (DTT), 0.15 mM NADH, 10 U phosphoglycerate kinase and 2 mM 3-phosphoglycerate as a substrate. The reactions were started with the addition of extract.

Phosphoglycerate kinase was measured in the reverse reaction in the presence of 0.9 mM EDTA, 0.15 mM NADH, 1 mM ATP and 1.6 U glyceraldehyde 3-phosphate dehydrogenase and 3-phosphoglycerate as substrate.

Phosphoglycerate mutase was assayed in 0.15 mM NADH, 1 mM ADP, 0.5 mM glycerate 2,3-bisphosphate, 0.9 mM EDTA, 2.8 U lactate dehydrogenase, 1.4 U pyruvate kinase, 0.6 U enolase and 3-phosphoglycerate as a substrate.

Enolase was measured with 0.15 mM NADH, 1 mM ADP, 0.9 mM EDTA, 2.8 U lactate dehydrogenase, 1.4 U pyruvate kinase, and 2-phosphoglycerate as a substrate.

Pyruvate decarboxylase was assayed with 5 mM MgCl$_2$ (replacing the MgSO$_4$ of the assay buffer), 0.2 mM thiaminepyrophosphate (TPP), 15 mM NADH, 22 U alcohol dehydrogenase and pyruvate as a substrate.

Alcohol dehydrogenase was assayed in the reverse direction with 1 mM of oxidized glutathione, 10 mM semicarbazide and 2 mM NAD with ethanol as the substrate.

For measurement of phosphofructokinase kinetics, the enzyme was partly purified from a cell free extract. The extract was prepared in extraction buffer containing 40 mM of MES, 100 mM KCl, 1 mM MgCl$_2$, 2 mM fructose 6-phosphate, 50 mM KH$_2$PO$_4$, 1 mM PMSF and 5 mM β-mercaptoethanol at pH 6.4. To 25 g yeast in 5 ml buffer, 25 g of glass beads was added and the cells were broken in a CO$_2$-cooled homogenizer (Braun). The extract was centrifuged and phosphofructokinase in the supernatant was purified by ammonium sulphate precipitation and Sephadex G200 chromatography using an eluent buffer containing 40 mM of MES, 100 mM KCl, 1 mM MgCl$_2$, 0.2 mM ATP, 25 mM KH$_2$PO$_4$ and 5 mM mercaptoethanol at pH 6.8. The assay contained 70 mM Pipes buffer at pH 7.0, 90 mM KCl, 10 mM MgSO$_4$, 15 mM KH$_2$PO$_4$, 10 mM NH$_4$Cl, 0.15 mM NADH, 0.3 U aldolase, 2.7 U triosephosphate isomerase and 0.5 U glycerol 3-phosphate dehydrogenase. The coupling enzymes were desalted over a PD10 column (Pharmacia). Fructose 6-phosphate, ATP, AMP and fructose 2,6-bisphosphate were used at various concentrations as substrates and or effectors.

For pyruvate kinase, cells were extracted with glass beads in 100 mM Pipes buffer of pH 7.0 containing 10 mM KCl and 1 mM PMSF. The assay contained 70 mM Pipes buffer at pH 7.0,
100 mM KCl, 1 mM MgCl\(_2\), 0.2 mM NADH, 2 U lactate dehydrogenase and ADP and PEP as variable substrates. The effect of fructose 1,6-bisphosphate on the kinetics of pyruvate kinase was tested in a concentration range of 0.025 to 5 mM.

**Experimental determination of metabolites and fluxes in the intact cells.**

We have also measured most glycolytic intermediates and fluxes in the compressed yeast Koningsgist from DSM Bakery Ingredients. The yeast cake was resuspended in potassium phosphate buffer (100 mM, pH 6.5), and mixed with pre-warmed (30 °C) and nitrogen-fluxed phosphate buffer to a final concentration of 50 g cake l\(^{-1}\) (with is 5-6 g l\(^{-1}\) protein, using Lowry's protein assay [224] with BSA as a standard). Glucose was added to a final concentration of 100 mM and samples were withdrawn at regular time-intervals and quenched in ice-cold perchloric acid or -40 °C methanol, depending on the metabolites. Most metabolites were measured in 5% (final concentration) perchloric acid extracts (G6P, F6P, F16bP, DHAP, NAD, ATP, ADP and AMP), which was also used to measure the pathway substrates and products glucose, ethanol, glycerol, pyruvate, acetate and succinate. Extracts were neutralized with potassium carbonate and assayed for the metabolites by enzymatic analyses according to Bergmeyer [25], as reported earlier [82, 299]. Intracellular pyruvate, PEP, 2PGA, 3PGA, NAD and NADH were measured by first quenching metabolism in cold methanol, which allows removal of the extra-cellular medium and concentration of the sample. After centrifugation of the methanol quenched cells, NADH was measured by alkaline extraction of the pellet according to [360]; the other metabolites were extracted again with perchloric acid and analyzed as described [299]. Metabolites, glucose, acetate and succinate were measured by enzymatic analysis; ethanol and glycerol were measured by HPLC (Aminex 87H column from Biorad, mobile phase 0.01 N \(\text{H}_2\text{SO}_4\) at 0.5 ml/min, refraction index detector 131 from Gilson). Acetaldehyde was determined by rapid filtration immediately after taking a sample, as described in [297]. Equilibration of intracellular and extracellular acetaldehyde was assumed. Carbon dioxide production was measured by off-gas analysis using mass spectrometry (VG gas analysis systems Ltd, type MM8-80), calibrated by air of known composition.

**8.4 Results**

*The intact cell: measurement of metabolites and fluxes.*

We have monitored glucose consumption and product formation for 45-60 minutes after addition of 100 mM of glucose to resuspended compressed yeast. Under the same conditions metabolite levels were measured. After addition of glucose, most of the intracellular metabolites reached a steady state within a few minutes (Fig. 2). Fructose 1,6-bisphosphate and ATP engaged in a slight transient oscillation but appeared to settle to a steady level at 30 min. Most fluxes
were constant throughout the experiment as evident from the variation of the concentrations (Fig. 3). Other fluxes showed more complex behavior, especially the net flux into trehalose, which was negative at first but became positive after some 20 minutes (Fig. 3). After 20 minutes all fluxes had reached a steady state, as had the metabolite concentrations (although some metabolite concentrations still showed minor time-dependences). In view of the variation of variables in the first 20 min, the rates and metabolite levels summarized in Table I were averaged over the period from 20 to 40 minutes after glucose addition.

The concentration of CO₂ in the off-gas showed an increase for the first 20 min after glucose addition, then a constant level for 20 min and a subsequent decrease as the glucose concentration in the medium depleted. The initial increase in CO₂ evolution is caused by equilibration of the buffer with bicarbonate and by the time required to fill the head space of the fermentor, until a steady state is reached after some 20 min. The constant level of CO₂ in the off-gas between 20 and 40 min after glucose addition was used to calculate the rate of CO₂ evolution.

In Table I it can be seen that most of the glucose is converted into ethanol and CO₂, but a significant proportion of the glucose-derived carbon is directed to the branches of glycolysis. These include glycogen, trehalose, glycerol and succinate. Small amounts of acetate and pyruvate were found. Since 1 mole of succinate derived from glucose produces 5 moles of reducing equivalents [329], the production of succinate can account for 80% of the glycerol
formed during the glucose fermentation. Together with the acetate and pyruvate produced, 95% of the glycerol formation could be accounted for in terms of the redox balance.

The rate of CO₂ production is higher than can be accounted for by the rates of ethanol and succinate production. We assume that the difference is caused by loss of ethanol (and possibly acetaldehyde) via the off-gas. If 1 mol of glucose is taken to produce 2 moles of CO₂, the carbon balance is closed for 90%.

Enzyme kinetics and displacement from equilibrium.

This paper addresses the question whether the enzyme kinetic parameters as determined in vitro can predict the in vivo fluxes and metabolite concentrations in the glycolytic pathway of S. cerevisiae. One might expect that the necessary data are all present in the literature. The literature data, however, were mostly determined for experimental conditions different from those of our intact-cells experiment. Therefore we determined a number of kinetic parameters in an extract of the cells also used for the flux and metabolite measurements. The literature was perused to select the best assay, to determine the kinetic mechanism, to prevent pitfalls and to complement our own data set. The result was a set of kinetic parameter values of all glycolytic enzymes for a single condition. Table II gives this, unique, parameter set. In Table III the maximum forward rate for each enzyme is given, together with the equilibrium constant of each reaction. A detailed discussion of the parameter values and the chosen mechanisms for each enzyme can be found in Appendix I. In Table III also the displacement from equilibrium is given for each step, using the experimentally determined metabolite concentrations and the equilibrium constants. The glucose transport step was lumped with the hexokinase step into one module. No data on the intracellular glucose concentration was (yet) available for the condition analyzed here. The same was done for the enzymes GAPDH and PGK. The concentration of the metabolite linking these enzymes, 1,3-bisphosphoglycerate, is unstable and is too low to measure accurately. The steps close to equilibrium were PGI, the combined GAPDH-PGK step and PGM. The transport-HK step and PYK were virtually irreversible. Also enolase and aldolase were quite far from equilibrium. Consequently, the widely used assumption that the lower part of glycolysis from F16bP to PEP is close to equilibrium cannot be made for our conditions.

Model results: unbranched glycolysis

At first, we modeled glycolysis without the branch reactions, arguing that for non-growing cells the flux through these branches is relatively small compared to the mainstream flux from glucose to ethanol. In a subsequent model (below) the branches will be introduced and their effects studied.
Figure 3. Fluxes during anaerobic glucose fermentation in resting yeast. A: glucose (■), ethanol (▲) and CO$_2$ evolution rate (○). B: glycerol (▲), succinate (○), acetate (■) and pyruvate (♦). C: glycogen (▲), trehalose (●) and acetaldehyde (○).

MATHEMATICAL MODEL OF YEAST GLYCOLYSIS
<table>
<thead>
<tr>
<th>Metabolites</th>
<th>concentration (mmol L cytosol(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>G6P</td>
<td>2.45 ± 0.14</td>
</tr>
<tr>
<td>F6P</td>
<td>0.62 ± 0.02</td>
</tr>
<tr>
<td>F16bP</td>
<td>5.51 ± 0.04</td>
</tr>
<tr>
<td>DHAP</td>
<td>0.81 ± 0.01</td>
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<tr>
<td>Gly3P</td>
<td>0.15 ± 0.10</td>
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<tr>
<td>3PGA</td>
<td>0.90 ± 0.02</td>
</tr>
<tr>
<td>2PGA</td>
<td>0.12 ± 0.01</td>
</tr>
<tr>
<td>PEP</td>
<td>0.07 ± 0.00</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>1.85 ± 0.64</td>
</tr>
<tr>
<td>Acetaldehyde</td>
<td>0.17 ± 0.02</td>
</tr>
<tr>
<td>ATP</td>
<td>2.52 ± 0.20</td>
</tr>
<tr>
<td>ADP</td>
<td>1.32 ± 0.10</td>
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<tr>
<td>AMP</td>
<td>0.25 ± 0.07</td>
</tr>
<tr>
<td>NAD</td>
<td>1.20 ± 0.13</td>
</tr>
<tr>
<td>NADH</td>
<td>0.39 ± 0.09</td>
</tr>
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Table I. Experimental determination of fluxes and metabolite concentrations in compressed yeast challenged with 100 mM glucose. See Materials and Methods for details.

\(\text{a)}\) included for comparison with specific activities of Table III.
<table>
<thead>
<tr>
<th>Enzyme</th>
<th>$K_a$ (mM)</th>
<th>$K_b$ (mM)</th>
<th>$K_p$ (mM)</th>
<th>$K_q$ (mM)</th>
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</thead>
<tbody>
<tr>
<td>Glc transport</td>
<td>1.19 (Glc$_{ext}$)</td>
<td>1.19 (Glc$_{in}$)</td>
<td></td>
<td></td>
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<tr>
<td>int. const.</td>
<td>0.91</td>
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<tr>
<td>HK</td>
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<td>0.23 (ADP)</td>
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<td>PGI</td>
<td>1.4 (G6P)</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>ALD</td>
<td>0.3 (F16bP)</td>
<td></td>
<td>2.4 (DHAP)</td>
<td>2.0 (GAP)</td>
</tr>
<tr>
<td>$K_i$</td>
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<td></td>
<td>10 (GAP)</td>
<td></td>
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<tr>
<td>GAPDH$^x$</td>
<td>0.21 (GAP)</td>
<td>0.09 (NAD)</td>
<td>9.8 \cdot 10^3 (13PGA)</td>
<td>0.06 (NADH)</td>
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<td>0.3 (ATP)</td>
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<td></td>
<td></td>
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<td>Hill coefficient</td>
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<td>0.17 (NAD)</td>
<td>0.11 (NADH)</td>
<td>1.11 (AcAld)</td>
</tr>
<tr>
<td>$K_i$</td>
<td>90 (EtOH)</td>
<td>0.92 (NAD)</td>
<td>0.031 (NADH)</td>
<td>1.1 (AcAld)</td>
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<tr>
<td>G3PDH</td>
<td>0.4 (DHAP)</td>
<td>0.023 (NADH)</td>
<td>1 (G3P)</td>
<td>0.93 (NAD)</td>
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</table>

<table>
<thead>
<tr>
<th>PFK$^{(a)}$</th>
<th>$K_R$ (mM)</th>
<th>$c$</th>
<th>$K$ (mM)</th>
<th>$c_i$</th>
</tr>
</thead>
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<tr>
<td>F6P</td>
<td>0.1</td>
<td>0</td>
<td>0.65</td>
<td>100</td>
</tr>
<tr>
<td>ATP</td>
<td>0.71</td>
<td>3</td>
<td>0.0995</td>
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<td>AMP</td>
<td></td>
<td></td>
<td>0.111</td>
<td>0.397</td>
</tr>
<tr>
<td>F16bP</td>
<td></td>
<td></td>
<td>6.82 \cdot 10^4</td>
<td>0.0174</td>
</tr>
<tr>
<td>F26bP</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>other</td>
<td>5.12 ($g_r$)</td>
<td>1 ($g_i$)</td>
<td>0.66 ($L_o$)</td>
<td></td>
</tr>
</tbody>
</table>

Table II. Kinetic parameters used in the model. The source of each parameter is given in Appendix I. See model description for the rate equations used for each enzyme.

$^{(a)}$ For equation and meaning of the symbols, see Appendix II
An important consequence of the stoichiometry of unbranched glycolysis is that the concentration of oxidized metabolites in the lower part of glycolysis and that of NAD are subject to a redox conservation relationship (see Eq. 8.3). Using the experimentally determined metabolite levels, this conserved sum of oxidized substances was calculated at 4.3 mM. For the other two conserved moieties $\Sigma_i$ and $\Sigma_2$ (Eq. 8.1 and 8.2), values were calculated at 4.1 and 1.6 mM, respectively.

Using these moiety conserved sums and the parameter values given in Table II, we numerically integrated the differential equations. The steady state concentrations of the metabolites were used as initial conditions (Table I). Earlier models of the glycolytic pathway exhibited various types of dynamic behavior ranging from no steady state at all, stable steady states [78, 108] through limit-cycle oscillations [303] to deterministic chaos [40]. Our experiments (Figs. 2 and 3) suggested that a steady state should be reached. We first checked whether the in vitro kinetics predicted such a (stable) steady state. No steady state was reached with this set of model parameters.

Inspection of the time course of the simulation showed that F16bP, GAP and DHAP were continuously increasing to extremely high levels, whereas the other metabolites reached a constant level. The flux through the enzymes upstream F16bP reached a constant level of 0.48 C-mol min$^{-1}$ L cytosol$^{-1}$, whereas the flux through the enzymes downstream GAP reached a level of only 0.38 C-mol min$^{-1}$ L cytosol$^{-1}$, explaining the accumulation of the metabolites in between. This failure of the “lower” part of glycolysis to keep up with the flux through the “upper” part is reminiscent of the phenotype seen in mutants in trehalose 6-phosphate synthase (Tps1) [129, 363] (chapter 6). Tps1 mediates a negative feedback on the hexokinase step in an as yet unknown manner (see [363] and Appendix I). This feedback is absent from the model used here, and this result may therefore have been expected.

The appearance of a “tps1Δ-phenotype” requires a limitation in the lower part of glycolysis (chapter 6). Even though all $V_{\text{max}}$-values downstream aldolase exceeded the $V_{\text{max}}$ of glucose transport (Table III), this limitation can be understood by the moiety conservation of the oxidized species in the lower part of glycolysis (Eq. 8.3). The calculated pyruvate concentration was about two times higher than measured (Table I): 4.0 mM rather than 1.85 mM (data not shown). As the sum of the concentrations from 13PGA to acetaldehyde, plus NAD, cannot exceed 4.3 mM (i.e. $\Sigma_3$), the higher level of pyruvate should lead to lower concentrations of the other members of the conserved sum. Consequently, the NADH/NAD ratio attained a very high level of 8.1. At this high NADH/NAD ratio, the rate of GAPDH is severely inhibited: the effective $V_{\text{max}}$ of GAPDH was decreased by a factor of 2. At the concentration of 13BPG required for sufficient PGK activity, the concentration of GAP, the substrate of GAPDH, could not increase sufficiently as to enable GAPDH to proceed at the required flux of 0.48 C-mmol min$^{-1}$ L cytosol$^{-1}$. 
Enzyme | $V_{\text{max}}$ (U mg protein$^{-1}$) | $K_{\text{eq}}$ | $\Gamma/K_{\text{eq}}$ | Reference
---|---|---|---|---
Glc transport | 0.36 | 1 | 6.75 · 10$^{-6}$ | [349]
HK | 0.84 | 3.8 · 10$^3$ | | |
PGI | 1.26 | 0.314 | 0.806 | [359]
PFK | 0.68 | | | |
ALD | 1.19 | 0.069 | 0.078 | [25]
TPI | 8.4 | 0.045 | 1 | [25]
GAPDH | 4.4/24.3 (a) | 0.0056 | 0.877 (b) | [59]
PGK | 4.8 (c) | 3.2 · 10$^3$ | | [25]
PGM | 9.4 | 0.19 | 0.703 | [131]
ENO | 1.35 | 6.7 | 0.087 | [25]
PYK | 4.05 | 6.5 · 10$^4$ | 7.76 · 10$^3$ | [25]
PDC | 0.65 | | | |
ADH | 3.0 (d) | 1.45 · 10$^4$ | 0.531 | [55]
AK | 0.45 | 0.803 | | [25]
G3PDH | 4.3 · 10$^1$ | 1.33 · 10$^4$ | | |

Table III. Experimentally determined maximal rates, equilibrium constants from literature (as specified in the last column) and displacement from equilibrium for each step in the model. Maximal rates are expressed as U (i.e., μmol min$^{-1}$) per mg total protein. For aldolase the unit of the equilibrium constant is mM$^{-1}$. In cases where protons are involved, the concentration of H$^+$ was taken to be 10$^{-7}$ M (pH 7.0). $\Gamma$ is the mass-action ratio ([products]/[substrates]), based on the steady-state metabolite concentrations of Table I.

(a) Combined mass action and equilibrium constant of glucose transport and hexokinase. The equilibrium constant of hexokinase was calculated from standard thermodynamic properties of the reactants from [4].
(b) Combined mass action and equilibrium constant of GAPDH and PGK.
(c) The $V_{\text{max}}$ for the forward and reverse reaction are given, respectively.
(d) For PGK and ADH the $V_{\text{max}}$ for the reverse reaction was measured. For ADH the equilibrium constant is also defined in the reverse reaction (acetaldehyde and NADH being the products).

To check whether a stable steady state could be reached if the pyruvate concentration were correctly predicted, the maximal rate of PDC was increased until the proper pyruvate concentration was reached. A 6.1-fold increase in the $V_{\text{max}}$ of PDC was required. With this adjustment, a stable steady state was reached. Table IV shows the model prediction in this case. The glucose consumption rate was 0.33 μmol min$^{-1}$ mg protein$^{-1}$, which was some 20% lower than the measured value of 0.40 μmol min$^{-1}$ mg protein$^{-1}$. Most of the calculated metabolite levels did
not differ by more than a factor of 2 from the experimental values. Exceptions are F6P, F16bP and NADH.

Is the correspondence between in vitro kinetics and in vivo behavior improved by considering the branches of glycolysis?

An alternative explanation for the failure of the original in vitro kinetics to describe all aspects of the in vivo situation could be that the branches from glycolysis were ignored. Indeed, although each individual flux through the branches into trehalose, glycogen, glycerol and succinate was small compared to the flux to ethanol, their combined effect resulted in a significant reduction in the ethanol flux as compared to the theoretical maximum (0.57 μmol min⁻¹ mg protein⁻¹ (based on CO₂ evolution) and 0.80 μmol min⁻¹ mg protein⁻¹ (based on the rate of glucose consumption), respectively). Therefore, we have introduced those branches and examined their effect on the metabolite levels.

The kinetics of the enzymes of most branches are not well characterized. The branches into trehalose and glycogen were introduced as constant rates and did therefore not require additional parameter estimation. This is different for the glycerol and the succinate branch, because introduction of these fluxes as constants would maintain the moiety conservation of oxidized species (Σx).

The glycerol branch was modeled as being controlled by glycerol 3-phosphate dehydrogenase, the first step in the pathway. There is some kinetic information of that enzyme in the literature [6]. Our attempts to measure the limiting rate experimentally, however, were hampered by a strong salt and protein concentration dependency of the G3PDH activity (see Appendix I). The Vmax was therefore tuned to fit the metabolite levels and the measured flux. A similar approach was used to model the succinate branch: k succinate (Eq. 8.36) was calculated to fit the flux and the measured acetaldehyde concentration.

The implications of the extended model are shown in Table IV. The maximal rate of PDC was kept at its original, measured, level. A stable steady state was reached: the introduction of the glycerol branch relieved the moiety conservation of oxidized compounds, so that the higher pyruvate concentration does not force the NAD concentration to be low. Indeed, the NADH/NAD ratio in the extended model is low and therefore GAPDH’s activity can reach the rate required for a steady state. As expected from the low fluxes through the branches, the metabolite levels were comparable with those of the unbranched version of the model, although they appear to be somewhat lower and more displaced from the measured values than in the unbranched case. Including the branches does not lead to a better correspondence between model prediction and experiment than including an increased activity of PDC. The branches, however, are in direct correspondence with what is known experimentally.
<table>
<thead>
<tr>
<th></th>
<th>Unbranched model</th>
<th>Branched model</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Fluxes</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(mmol min(^{-1}) L cytosol(^{-1}))</td>
<td></td>
</tr>
<tr>
<td>Glucose</td>
<td>88</td>
<td>88</td>
</tr>
<tr>
<td>Ethanol</td>
<td>176</td>
<td>129</td>
</tr>
<tr>
<td>Glycerol</td>
<td>-</td>
<td>18.2</td>
</tr>
<tr>
<td>Succinate</td>
<td>-</td>
<td>3.6</td>
</tr>
</tbody>
</table>

| **Metabolite concentrations** |                  |                |
|                             | (mmol L cytosol\(^{-1}\)) |                |
| G6P                         | 1.51              | 1.07           |
| F6P                         | 0.16              | 0.11           |
| F16bP                       | 0.98              | 0.60           |
| DHAP                        | 0.95              | 0.74           |
| 3PGA                        | 0.52              | 0.36           |
| 2PGA                        | 0.07              | 0.04           |
| PEP                         | 0.08              | 0.07           |
| Pyruvate                    | 1.85              | 8.52           |
| acetaldehyde                | 0.24              | 0.17           |
| ATP                         | 2.52              | 2.51           |
| ADP                         | 1.29              | 1.29           |
| AMP                         | 0.30              | 0.30           |
| NAD                         | 1.55              | 1.55           |
| NADH                        | 0.04              | 0.04           |

Table IV. Model predictions of steady-state fluxes and metabolite concentrations in the unbranched and branched model. Concentrations of glucose and ethanol were fixed at 50 mM. In both models, \(K_{ATPase}\) was tuned to obtain the measured ATP concentration of 2.5 mM. In the unbranched model, the maximal rate of PDC was adjusted to obtain the experimentally obtained concentration of pyruvate (1.85 mM). Without this adjustment, no steady state was reached (see text for explanation). In the branched model, the \(V_{\text{max}}\) of G3PDH was tuned to yield the measured glycerol flux and \(k_{\text{succinate}}\) was adjusted to obtain the measured concentration of acetaldehyde of 0.17 mM.
Assessing the difference between in vitro and in vivo kinetics for the glycolytic enzymes.

The observation that the in vitro determined kinetic parameters for the glycolytic enzymes did not enable us to calculate properly all the in vivo concentrations of glycolytic metabolites, shows that some of the kinetic parameters do not reflect the true in vivo kinetic properties of the enzymes. The next question that seems relevant is, how far away from the prediction is the in vitro biochemistry? Is the in vitro biochemistry for all enzymes remote from their in vivo behavior, or is there just a discrepancy for one or two enzymes? The fact that virtually all calculated metabolite concentrations differ significantly from the experimentally determined ones, does not prove that also the biochemistry of all enzymes is wrong. An error in one kinetic parameter may well affect the flux and all metabolite concentrations.

From the experimental flux and the reaction stoichiometrics, one can readily deduce the steady state rate of each enzyme. For most enzymes the concentrations of all its substrates, products and metabolic modifiers were measured as well. For each enzyme one can therefore examine whether these concentrations and the rate satisfy the rate equation of the enzyme. Moreover, for each enzyme one can calculate by how much one would have to change the magnitude of any of the parameters in order for the rate and concentrations to satisfy the rate equation.

Because the intracellular amount of the enzymes is often considered the most uncertain parameter, various authors have calculated the enzyme concentration that fitted the experimental data [305, 407]. Some authors then even used these calculated enzyme concentrations as the true concentrations in their model [305]. Here we refrain from deciding a priori which kinetic parameter is at fault. Rather, we ask for each enzyme by how much we would have to change: i) the $V_{\text{max,forward}}$ and $V_{\text{max,reverse}}$ (i.e., the intracellular concentration of the enzyme), ii) the equilibrium constant (through changing the maximal reverse rate, leaving all other kinetic constants unchanged), and iii) the substrate/product affinities (equally, keeping the equilibrium constant unchanged; when two substrates and two products were present, either the $K_m$'s for the structurally related substrate and product were changed in pairs, or those of two reactants whose affinity were most uncertain).

For the concentrations of intracellular glucose and 1,3-bisphosphoglycerate no data were available, and a steady-state assumption was used to lump the reactions around those metabolites. Thus, equating the rates of glucose transport and hexokinase resulted in a combined equation for the rate through these two steps as a function of the concentration of external glucose, ATP, ADP and glucose 6-phosphate only. Likewise, the combined rate of GAPDH and PGK was a function of the concentration of GAP, NAD, NADH, ATP, ADP and 3PGA. The result for the limiting rates, the equilibrium constant and the affinities for each enzyme/enzyme combination is shown in Table V.
Changes in affinity constants did not always lead to a solution within the constraints of the Haldane relationship. Where a solution was found, however, the changes required were generally much larger than for the limiting rate or the equilibrium constant (Table V).

The $V_{\text{max}}$ of glucose uptake was 10% lower than the flux through that step. Consequently, no solution was found for any kinetic parameter in the transport-HK module except for the maximal rate of glucose transport. For half of the other enzymes the in vitro $V_{\text{max}}$ needed adjustment by less than a factor of 2 for the model to predict the in vivo rate at the experimentally determined metabolite levels (Table V).

For the other enzymes the discrepancy between in vitro kinetics and in vivo rate was larger. The $V_{\text{max}}$ of PDC had to be increased some 6-fold; changes in its other parameters did not lead to a solution. The kinetics of PGI had to be changed some 4-fold, either by an increase in its maximal rate or by increasing its equilibrium constant. Aldolase required a 3-fold reduction in its limiting rate, or an 8-fold decrease in its equilibrium constant. Alcohol dehydrogenase was also about three times more active in vitro than it should be in vivo.

The solution for the GAPDH/PGK module is more complicated because of the uncertainty in the concentration of 13PGA. The overall equilibrium constant is about 17, but it is the product of a very small equilibrium constant for GAPDH (0.0056 at pH 7 and a phosphate concentration of 10 mM) and a very large one for PGK (3200). Therefore, the concentration of 13PGA should be extremely small, and apart from its instability, is not measurable. The mass action ratio of the substrates and the products of this combined step are close to the overall equilibrium constant (Table III). Consequently, both reactions must be close to equilibrium for both reactions to proceed in the forward (glycolytic) direction. This constraint allows only a very small range of 13PGA concentrations: $5.37 \cdot 10^4 < [13PGA] < 6.28 \cdot 10^4$ mM. With the kinetics of GAPDH, no concentration of 13PGA could be found that would lead to the required flux through the enzyme. With the kinetics of PGK, the concentration of 13PGA required to match the flux is $7.76 \cdot 10^4$ mM, higher therefore than is allowed for the GAPDH reaction to proceed in the glycolytic direction. Therefore, simultaneous adjustment of kinetic parameters of both enzymes is required. This has been examined for three cases: i) $[13PGA] = 5.4 \cdot 10^4$ mM, i.e. GAPDH is close to equilibrium, ii) $[13PGA] = 5.83 \cdot 10^4$ mM, i.e. both enzymes are equally displaced from equilibrium, and iii) $[13PGA] = 6.2 \cdot 10^4$ mM, i.e. PGK is close to equilibrium. For GAPDH, solutions were calculated for adjusting the $V_{\text{max}}$ in both directions or for changing the $V_{\text{max}}$ in the forward direction only; the latter adjustment changes the equilibrium constant. Other parameter changes did not lead to a solution. For PGK calculations were done for the $V_{\text{max}}$ of PGK in both directions, or for an adjustment of the $K_m$ of PGK for 13PGA (the most uncertain kinetic parameter for PGK). The latter manipulation also changes the equilibrium constant. Table VI summarizes the solutions in each case. A change in the equilibrium constants, via alterations in the forward rate of GAPDH and the $K_m$ of 13PGA for PGK, was the most effective way to match the in vitro kinetics with the in vivo behavior. This was to be expected for enzymes close to
equilibrium. Changes in maximal rates are much less effective for enzymes close to equilibrium. Consequently, large changes in the maximal rates of GAPDH and or PGK were required for each assumed concentration of 13PGA.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>$V_{\text{max}}$</th>
<th>$K_{\text{eq}}$</th>
<th>$K_a/K_p$</th>
<th>$K_b/K_q$</th>
<th>$K_p/K_q$</th>
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<tbody>
<tr>
<td>HXT</td>
<td>1.2</td>
<td>-</td>
<td>n.s.</td>
<td>-</td>
<td>-</td>
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<tr>
<td>HK</td>
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<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td>PGI</td>
<td>4.1</td>
<td>3.8</td>
<td>n.s.</td>
<td>-</td>
<td>-</td>
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<td>PFK</td>
<td>0.73</td>
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<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td>ALD(a)</td>
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<td>0.12</td>
<td>28</td>
<td>37</td>
<td>109</td>
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<tr>
<td>PGM</td>
<td>1.0</td>
<td>1.0</td>
<td>0.96</td>
<td>-</td>
<td>-</td>
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<tr>
<td>ENO</td>
<td>0.73</td>
<td>0.26</td>
<td>11</td>
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<td>-</td>
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<td>PYK(b)</td>
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<td>n.s.</td>
<td>0.89</td>
<td>0.65</td>
<td>1.4</td>
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<td>PDC(c)</td>
<td>6.1</td>
<td>-</td>
<td>n.s.</td>
<td>-</td>
<td>-</td>
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<tr>
<td>ADH</td>
<td>0.11</td>
<td>6.0</td>
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<td>-</td>
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</table>

Table V. Required adjustments to $V_{\text{max}}$, $K_{\text{eq}}$, or pairs of Michaelis-Menten constants for each enzyme to fit the flux and measured metabolite concentrations for the branched model. Numbers in brackets give the factor by which the original parameter had to be multiplied.

"-" : not calculated
"n.s." : no solution

(a) $K_a/K_p$ corresponds to F16bP and GAP; $K_b/K_q$ corresponds to F16bP and DHAP; $K_d/K_q$ corresponds to GAP and DHAP, where $K_p$ should be multiplied and $K_q$ divided by the indicated factor.

(b) $K_w$, $K_o$, $K_a$ and $K_b$ correspond to PEP, ADP, Pyr and ATP, respectively. For $K_w/K_q$, $K_o$ should be multiplied and $K_q$ divided by the indicated factor.

(c) $K_d/K_q$ stands for the $K_{0.5}$ of pyruvate only.

8.5 Discussion

The model described in this paper is a first attempt systematically to test our biochemical knowledge of the glycolytic enzymes of yeast to describe \textit{in vivo} behavior of the whole pathway. The analysis has pinpointed some specific weak spots in our current biochemical knowledge. These will be discussed in more detail below; first we will explain the differences between our model and the ones found in literature.
Comparison of models: different questions, different approaches

Quite a few mathematical models of yeast glycolysis do already exist, e.g. [77, 78, 108, 303, 305, 323]. The approach in the existing models has been quite different compared to ours. In the biotechnological context, validation of a model implies fitting the model equations to the experimental data: the model is satisfactory if a good fit is obtained. This was the context of the modelling by Galazzo et al [108], and Rizzi et al. [305]. The objective is a model that can describe the *in vivo* behavior and is hopefully able to predict this behavior under some other conditions. However, many rate equations, also mechanistically incorrect ones, may give good fits. This may not matter for some biotechnological applications and is perhaps unavoidable for some enzymes (phosphofructokinase, for example), but from a fundamental biochemical view it is unsatisfactory.

An example should illustrate the point. Based on the transient changes in extracellular glucose and intracellular glucose 6-phosphate concentrations following a glucose pulse to a glucose-limited culture of *S. cerevisiae*, a good fit was obtained for the kinetics of the glucose transport step [306]. The fitted kinetic rate equation was based on competitive inhibition of glucose uptake by glucose 6-phosphate. The internal glucose concentration was ignored, assuming that it was extremely low in glucose-consuming yeast cells. However, in chapter 4 it was shown that the internal glucose concentration may be as high as 1.5 mM. This concentration of intracellular glucose was high enough to explain the negative feedback regulation of high affinity glucose transport by internal glucose rather than glucose 6-phosphate (chapter 4). The correlation between glucose 6-phosphate and glucose uptake activity is not unique [274, 384], and the observed pattern in [306] may be explained by an effect of glucose 6-phosphate on the intracellular glucose concentration through inhibition of hexokinase. This example illustrates the limitation of fitting: a good fit does not necessarily ensure a mechanistically correct description of the process. This may not matter so much as long as the phenomenological model

<table>
<thead>
<tr>
<th>[13PGA] (μM)</th>
<th>[E\text{GAPDH}]</th>
<th>V^+\text{GAPDH}</th>
<th>[E\text{PGK}]</th>
<th>K_{m,13PGA}</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.540</td>
<td>11.7</td>
<td>2.50</td>
<td>72.8</td>
<td>0.70</td>
</tr>
<tr>
<td>0.583</td>
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<td>2.58</td>
<td>5.1</td>
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</tr>
<tr>
<td>0.620</td>
<td>128.8</td>
<td>2.64</td>
<td>2.8</td>
<td>0.80</td>
</tr>
</tbody>
</table>

Table VI. Required adjustments to kinetic parameters of GAPDH and PGK for each enzyme to fit the flux and measured metabolite concentrations for the branched model, at different concentrations of 13PGA. See text for explanation.
is used for interpolation between experimental data points, but it increases the chance of erroneous prediction when the model is used outside those experimental boundaries.

An alternative to fitting is to use the biochemical knowledge, gathered by in vitro kinetic studies, to predict the system behavior and then compare the prediction with experimental data. The prediction of the steady-state flux and the concentrations of the metabolites can be used to check whether the model makes sense or not. This approach was successful for the relatively simple glycolytic pathway of Trypanosoma brucei (see [14]), but less so for the much more complex TCA cycle of D. discoideum [407]. This approach was also used here: rather than a priori assuming that in vitro kinetics are of no use for modeling in vivo behavior, the comparison between the data sets may pinpoint to important parameters that can directly be related to, and assessed by, experimental data. In such an approach, the model is used as an important heuristic tool.

Realistic modeling of a pathway as complex as glycolysis is very difficult. Even when all biochemical knowledge of the glycolytic enzymes is collected, difficult choices and simplifications are required. It is also here that we have made some different choices compared to other models.

An important aspect of our model is the use of reversible kinetics for all but two enzymes (PFK and PDC). In a reaction sequence, the role of the product is as important as the role of the substrate. Even when an enzyme catalyzes an irreversible reaction, it may still be sensitive to its products. Product insensitivity leads to diminished control by the enzymes downstream the product-insensitive enzyme. In chapter 7 it was shown that this may be the case even if there is still a feedback by cofactors to which the enzyme is sensitive. The crucial role of product inhibition was clearly demonstrated in the case of hexokinase. The impact of the absence of Tps1-mediated feedback on hexokinase in our model was apparent in the unbranched model and will be discussed shortly.

Another difference with other models is that concentrations of the nicotineamine nucleotides have been fixed in all models but two [303, 305]. We have treated the concentrations of NAD and NADH as free metabolic variables, as they may play an important role in the regulation of glycolysis. They certainly are important in the regulation of the dynamics of yeast glycolysis, considering the important role of acetaldehyde in synchronizing glycolytic oscillations in populations of intact yeast cells [297, 298]. When branching occurs, as it does (Table I), the NADH/NAD ratio is important as it mediates the constraint imposed on these branches by the redox balance requirement.

Finally, our approach has been unique in that it has explicitly addressed the difficulty of combining and comparing kinetic parameters reported in the literature. Differences in assay conditions such as pH, temperature, buffer and ionic strength are obviously important, but the physiological state of the yeast used to make the extract and/or enzyme preparation should also
be taken into account. Many glycolytic enzymes have gluconeogenetic counterparts that have markedly different kinetic properties (e.g. the substrate affinity of glucose transport [292, 383] and alcohol dehydrogenase [112] can differ more than one order of magnitude, depending on the glucose concentration in the medium). Despite these difficulties, in most of the existing models, the rationale behind the choices for certain kinetic parameters from the many alternatives in the literature has not been discussed. Only Reuss’ group addressed this problem explicitly [305], but without going into any detail. To tackle these problems, we have measured the kinetic parameters for most glycolytic enzymes under the same assay conditions and from the same source. Although the set is necessarily incomplete, it was used as a guide through the labyrinth of kinetic parameters scattered in the literature, allowing us to pick the set that most closely fitted our conditions. Our set of kinetic parameters is therefore unique among the existing models in that each parameter choice is explicitly discussed in Appendix I.

Much effort was put in finding a proper rate equation for PFK. Our rate equation is the first one that takes F16bP inhibition into account. Even though the inhibition by F16bP may not be very effective under the conditions used, not putting F16bP inhibition in at all may itself confine much of the control over the glycolytic flux to the enzymes upstream of the compound. If such a confinement of control subsequently comes out of a model study (e.g. [77]), it may reflect more the properties the modeler has put into the model than those of true glycolysis. The inclusion of inhibition of PFK by F16bP is also an important improvement of the existing rate equations when the analysis is extended to other conditions and when studying dynamics of glycolysis. During glycolytic oscillations, for example, the concentration of F16bP does oscillate in a range where PFK is sensitive to this product [299] (chapter 3). Moreover, our equation may be used when studying the effect of F26bP metabolism on glycolysis, e.g. on its transition time during a transition from ethanol to glucose [45].

Modeling as a heuristic tool: limits in our biochemical knowledge of glycolytic enzymes

The calculation of the adjustments that were required to fit the kinetics to the observed in vivo behavior, showed that no limited set of errors in enzyme parameter values can be identified that could be responsible for the discrepancy between model and experiment. The kinetics of all enzymes required adjustment, albeit to various degrees.

The kinetics of enzymes may be affected by many factors whose concentration is different in the cell than in the assay. Moreover, unknown enzyme modifiers may exist which may change the apparent affinity constants significantly. Although fundamental equilibrium constants are well defined, the effective equilibrium constants used here may be affected to various extents through uncertainties in the exact activity of divalent cations (most notable Mg$^{2+}$), protons, phosphate and differences in temperature (our experiments being done at 30 °C whereas many equilibrium constants have been determined at 25 °C). The equilibrium constant can be corrected for the temperature difference via the Gibbs-Helmholtz equation, but this
requires knowledge of the standard enthalpy of the reaction, which is not known for all the
glycolytic steps and which is, once again, dependent on the concentration of divalent cations
and pH [4]. We have been able to make this correction for PGI. The equilibrium constant changed
from 0.29 at 25°C to 0.31 at 30°C, due to a rather large positive reaction enthalpy of 11.4 kJ mol
\textsuperscript{-1} [4, 359]. For other reactions this correction was not possible because data was lacking.
Therefore, it is not a priori obvious that only in vitro $V_{\text{max}}$ values are prone to error. We have
therefore not only calculated the adjustments in $V_{\text{max}}$ required to match in vitro and in vivo
activity, but also those in the effective equilibrium and affinity constants.

Most enzymes in the model required adjustments that appear reasonable given the
uncertainties in extraction efficiency of both metabolites and enzymes, the exact conditions in
the cytosol and errors in the determination of kinetic parameters. Some enzymes, however,
showed larger discrepancies than others.

The in vitro activity of ADH exceeded the in vivo flux through the enzyme by a factor of
ten. An uncertainty may be the presence of isoenzymes of ADH. We have estimated that ADHI
contributed to some 90% of the total activity of ADH, and used the kinetics for that isoenzyme in
the model (see Appendix I). Despite the markedly different kinetics of ADHI and ADHII, however,
incorporation of the kinetics for ADHII rather than for ADHI in the branched model did not
improve the model predictions significantly (results not shown). The large discrepancy between
in vitro and in vivo activity may be caused by the uncertainty in the free concentration of
cytosolic NADH. A large proportion of NADH may be bound and not available for ADH.
Moreover, our experiments did not discriminate between cytosolic and mitochondrial NADH.
The latter may contribute significantly to the total NADH pool, especially because under
anaerobic conditions the mitochondria may be expected to be reduced. The steady state
concentration of NADH required to match the in vitro ADH kinetics with a concentration of
acetaldehyde of 0.17 mM and the in vivo rate, was 0.04 mM, i.e. only 10% of the measured total
concentration of NADH. Such a low free NADH concentration would also fit much better with the
GAPDH kinetics. At 0.04 mM NADH, the concentration of GAPDH would have to be increased by
only a factor of 1.4, PGK remaining unchanged, cf. Table VI. It will therefore be very useful to
determine the free concentration of cytosolic NADH.

PDC required a sixfold increase in its maximal rate. Also Rizzi found difficulties with this
enzyme [305]. PDC is known for its dependence on phosphate. This concentration is as yet
uncertain. The values of $K_0$ and the Hill coefficient were taken from Boiteux [41], who measured
these values at a phosphate concentration of 25 mM. If the $K_0$ value for 5 mM phosphate is
taken (i.e. $K_0 = 2.2$ mM rather than 4.3 mM), a 2.4-fold increase in $V_{\text{max}}$ is required. Because of the
sensitivity of the calculation for the exact $K_0$ and Hill-coefficient, the kinetics of PDC in the
strain used in this study should be evaluated at the phosphate concentration prevailing in the
cytosol (which should be measured).
The in vitro activity of PGI was 4 times too low compared to the in vivo rate. The reason for this discrepancy is unclear. The measured kinetics fit reasonably well with the equilibrium constant via the Haldane relationship. The concentrations of G6P and F6P are close to their expected equilibrium concentrations, i.e. \([\text{F6P}] / [\text{G6P}] = 0.25\). This finding is in good agreement with earlier studies in resting yeast cells [23, 409]. A discrepancy for PGI between the in vitro and in vivo activity was reported earlier [23]. In that study, the in vitro PGI activity, calculated with the measured \(V_{\text{max}}\) and concentrations of G6P and F6P, was also some 4 times lower than the in vivo flux through the enzyme. The authors proposed the possibilities of channeling between PGI and PFK, and regulation of PGI by unknown mechanisms [23]. Alternatively, the measured concentrations of G6P and F6P may deviate from the true in vivo concentrations. Especially F6P appears sensitive to the extraction method and may differ significantly between different methods (chapter 1). A twofold decrease in the concentration of F6P would be required to match the in vitro and in vivo kinetics.

Aldolase appeared to be too active in vitro. Aldolase has been extensively studied in mammalian systems, but less so in yeast. Although most studies agree on the \(K_m\) of F16bP for this enzyme in yeast [17, 244, 302], not much information was found on the inhibition of the enzyme by the products GAP and DHAP. No data at all was found on the inhibitory constant of GAP \((K_i\) in Eq. 8); the value taken from Richter was an assumption only [303]. The value of \(K_i\) that would fit with the \(in vivo\) data is about three orders of magnitude smaller than the one estimated by Richter (Table IV). Complex formation of aldolase with G3PDH [377], GAPDH [262] and PFK [364] have been suggested. Polyethylene glycol (PEG), a macromolecule enhancing macromolecular crowding, affected the association of aldolase with G3PDH, with altered kinetic properties of yeast aldolase as a result [377]. The implication of such complex formation remains a matter of debate, but may include the inability of in vitro kinetics of aldolase to describe the in vivo rate through the enzyme.

It is interesting that the maximal activity of aldolase required to match the in vivo flux is close to that flux. This is caused by its substantial displacement from equilibrium and by the high affinity of aldolase for F16bP compared to the steady state concentration of F16bP, which are 0.3 and 5.5 mM, respectively. This means that the aldolase enzyme is largely saturated with its substrate. The rather peculiar situation arises, that both PFK (see below) and aldolase are hardly sensitive to the concentration of F16bP. Consequently, the concentration of F16bP can vary enormously without much effect on the rest of metabolism. Indeed, F16bP tends to show the largest variation in concentration upon addition of glucose to resting cells (Fig 3, see also e.g. [23]).

The last step that requires discussion is the combined GAPDH/PGK step. Specific difficulties arose from this module being close to equilibrium. Although GAPDH has been studied extensively, not many kinetic parameters under physiological conditions have been reported. Many studies were carried out at high pH where cooperative binding of NAD(H) is
observed (for review, see [139]). GAPDH is probably the most difficult enzyme in glycolysis to assay (see Appendix I). Indeed, the reported kinetics by Lambeir et al [211], also used by us, did not fulfill the Haldane relationship for the equilibrium constant. With our measured forward and backward maximal rates, the equilibrium constant calculated with the Haldane relationship was 5 times larger than the one measured by [59]. If the forward and backward maximal rates from Lambeir were used, the calculated equilibrium constant was even 75 times larger. For the kinetics of the glycosomal GAPDH from Trypanosoma brucei, this number was only 8 [211]. Thus, the kinetics of GAPDH should be handled with caution.

The adjustment of the equilibrium constant of GAPDH by a factor 2.5 is not unreasonable with the uncertainty in the phosphate concentration in the cell. A phosphate concentration of 25 mM rather than the 10 mM assumed for the equilibrium constant, would account for this factor 2.5. For a better comparison of the in vitro and in vivo rates, this concentration of phosphate should be determined.

PGK has been studied much more extensively under physiological conditions. Accordingly, the adjustments required for this enzyme are much smaller than for the GAPDH enzyme. The adjustment of the equilibrium constant for PGK is small. Considering the fact that the concentration of 13PGA is not measurable, a phenomenological description of the GAPDH-PGK module rate under a variety of product and substrate concentrations may suffice to model this step.

**Impact of enzyme characteristics on the systemic behavior of the model**

It is clear from the comparison of the maximal rates of all steps in the model, that glucose transport has by far the lowest $V_{\text{max}}$. Furthermore, the activity as measured over the first 5 s after addition of labeled glucose (the zero trans-influx rate) was somewhat lower than the actual glucose consumption rate. Moreover, hexokinase is hardly sensitive to its product G6P ($K_m = 30$ mM). These two features caused the glucose transport to control the glycolytic flux in both the unbranched and the branched model for some 90% (data not shown). Although it could be that indeed glucose transport is rate-controlling to such a large extent, there are two reasons to question such a conclusion.

First, as analyzed in chapter 5, high glycogen and trehalose levels may interfere with the determination of the initial uptake kinetics. The concentrations of these carbohydrates before addition of glucose were very high, comparable to the concentrations found after glucose-inactivation in chapter 5. This is not unexpected, as the trehalose concentration is a key parameter in determining shelf life of yeast, an important selection criteria for industrial Baker’s yeasts. Thus, the maximal rate of glucose transport may have been underestimated, following the reasoning of chapter 5.

Second, the model did not include a Tps1-mediated feedback on hexokinase (only weak G6P inhibition was included). If such a strong feedback were included, the steady-state rate of
hexokinase should decrease. This should lead to an increased steady-state intracellular glucose concentration and hence, to reduced steady-state glucose transport activity. Thus, the measured transport kinetics and the measured steady-state glucose consumption rate are incompatible with the presence of a strong feedback mechanisms, via Tps1, acting on hexokinase. The requirement for such a feedback mechanism has been amply described in this thesis. In the current model this requirement became apparent in the unbranched model with the original kinetics; a limitation in the GAPDH reaction was not signaled to the upstream reactions.

The model is therefore prone to metabolic imbalances similar to the ones seen in mutants deleted in the Tps1-mediated feedback on hexokinase. As shown in chapters 6 and 7, accumulation of intermediates can occur if (i) the ATP consuming steps (HK and PFK) are not sensitive to their carbon-derived products and (ii) if there is some limitation in the steps producing ATP. Inspection of the PFK kinetics (Appendix II) at the steady concentrations of the other substrates, products and effectors showed that indeed PFK is virtually insensitive to F16bP at F16bP concentrations above 5 mM (see Fig 7). Thus, at the high concentrations of F16bP that prevail during a high flux in the glycolytic reaction, PFK is hardly sensitive to its product. The consequence is that most control of the glycolytic flux resides at PFK and the upstream reactions.

The latter condition (a limitation in the ATP-producing lower part of glycolysis) was met in the case of the unbranched model by the inhibitory action of the high NADH/NAD ratio on GAPDH. In the branched model the NADH/NAD ratio was low, however, and no such limitation was present. Therefore, no accumulation of intermediates was seen.

Clearly, the rate of PFK should not exceed the capacity of the downstream reactions. This can be assured by limiting glucose uptake by the transporter, or by introducing a brake on hexokinase via Tps1. Regulation of hexokinase by Tps1 was also absent from all other models described so far, even though they all have rate equations for PFK that render the enzyme insensitive to F16bP. One reason that those models did not show a tps1-D phenotype may be that the need for product inhibition of hexokinase was bypassed by supposed glucose 6-phosphate inhibition of the glucose transport step [77, 78, 108, 305].

The insensitivity of PFK for its direct product F16bP has profound implications for the model. This emphasizes the importance of more detailed studies on the inhibition of PFK by PEP and citrate. So far, we haven’t found data on yeast PFK that would allow us to incorporate those effectors in our PFK-model. An early study seems to indicate that PEP has no large effect, but this study dates from before F26bP was discovered [28]. It is interesting to note, however, that even though the lower part of glycolysis may not be able to control the flux, it can be crucial for the control of dynamic behavior, such as oscillations (see also chapter 2). Thus, it is possible for ADH to have insignificant control on steady state variables, but to play a central role in the synchronization of glycolytic oscillations in populations of yeast cells [297].
In the model another enzyme was not sensitive to its product: PDC. Acetaldehyde sensitivity of PDC is impossible to assay using the conventional spectrometric method, because the assay is coupled to ADH. The implications of acetaldehyde insensitivity of PDC, however, are not so profound as those for the insensitivity of PFK for F16bP. The reasons differ for the branched and unbranched model. In the unbranched model, the conservation of oxidized species ensures that the enzymes upstream and downstream PDC are coupled, even if this is not through acetaldehyde. In the branched model, communication between the enzymes upstream and downstream PDC is mediated by the redox state, via redistribution of fluxes over the glycerol and succinate branches. As pointed out in chapter 7, moiety conservation and pathway branching are two mechanisms by which to reduce the effect of product insensitivity.

Consequences of stoichiometry: effect of adding branches to the model.

The introduction of branches in the model did not change the predicted metabolite concentrations to any striking extent. This is not unexpected considering the relatively small fluxes through the branches as compared to the ethanol formation flux. Is there any significant difference between the unbranched and branched model that would justify future effort in improving the rate equations of those branches? Yes, there is.

The most important consequence of introducing the glycerol and succinate branches has already been explained: it eliminates the moiety conservation of oxidized species in the lower part of glycolysis (Eq. 8.3). Compensation for an increase in the total amount of oxidized metabolites can now be given by a temporarily increase in glycerol production and or succinate production. Accordingly, introduction of these branches allows the sum of oxidized metabolites to vary. This has important consequences for the homeostasis of the redox state, which was illustrated by the change in the maximal rate of PDC. At the original kinetics of PDC, the NADH/NAD ratio was 8.1; a 6.1-fold increase in PDC activity led to a NADH/NAD ratio of only 0.023. The pyruvate concentration decreased as a result of the increased activity of PDC. Also other metabolites upstream of pyruvate decreased. Due to the conservation relationship $\Sigma$, the decrease in metabolites upstream of PDC had to result in an increase in the concentration of acetaldehyde and/or NAD (Eq. 8.3).

If we now consider ADH, it should be noted that its rate is really only determined by one independent variable, as acetaldehyde, NAD and NADH are linked through two moiety conservation relationships (Eqs. 8.2 and 8.3) and ethanol is considered a fixed external metabolite. Given the sum of acetaldehyde and NAD (as left from $\Sigma$) and the sum of NAD and NADH, there is a unique (positive) solution to the concentrations of those metabolites to sustain the proper flux through ADH. This solution is such that an increase in the sum of acetaldehyde and NAD as left from $\Sigma$, will lead to higher levels of both acetaldehyde and NAD, with a decrease of the NADH concentration as a result.
Any enzyme that can affect the concentrations of the metabolites partaking in the conservation relationship of oxidized species, therefore tends to control the redox state to a larger extent than would be expected without the moiety conservation. As a result, in the unbranched model the redox state is much more sensitive to changes in parameters than in the branched model. This can be quantified by the “total controllability” of, for example, NADH, defined as the sum of the absolute values of the NADH-concentration control coefficients (chapter 1). Indeed, the total controllability of NADH was 27 in the case of unbranched glycolysis and dropped to 2.0 when the glycerol and succinate branches were introduced. It should be noted that these numbers hold for the models with the original kinetics; for the unbranched model, PDC was adjusted to reach a stable steady state. For the optimized models, similar numbers were obtained (results not shown).

Interestingly, the control of the NADH concentration in the branched model was almost exclusively confined to those steps that have NADH and NAD as substrates or products (results not shown). In the unbranched model, all enzymes had large [NADH] control coefficients, depending on their ability to change the metabolites between GAPDH and PDC. Thus, the consequence of the presence of the glycerol and succinate branches is a more stable redox state, largely controlled by those enzymes that are closely involved in redox metabolism. Without these branches, the redox state would be very sensitive to perturbations in the activity of almost any glycolytic enzyme.

The glycerol branch is generally regarded merely as a redox sink that is required for oxidation of reducing equivalents during anaerobic growth [221]. Our analysis may suggest why even non-growing cells produce glycerol. The branch reactions may lead to dynamic buffering of the redox state. The seemingly wasteful simultaneous production of glycerol and succinate (and/or acetate [372]) may have this important homeostatic function.

Conclusions and perspectives

It is good modeling practice that experimentation and modeling follow each other: experiments falsify model assumptions, and model predictions suggest crucial experiments. We believe that such an iterative process will improve models and our understanding of metabolic pathways. This chapter reports on “a first round” in which in vitro kinetics of the enzymes of yeast glycolysis have been compared with the in vivo behavior of the pathway. It is clear that we are only at the beginning.

Our analysis has shown the impact of model limitations on the systemic behavior of the model. Realistic modeling of a metabolic pathway such as glycolysis is extremely difficult. All models of yeast glycolysis found in the literature so far have been based on fitting kinetic parameters to the observed metabolic variables. None of these models have been extensively validated after the fitting procedure. Some of the examples found in the literature, where control
analysis was performed based on glycolytic models, do not seem to have appreciated the difficulties associated with such an exercise, nor did they carefully discuss the implications of their model simplifications.

Our approach was to use modeling as a heuristic tool. The presented analysis has pointed at some uncertainties that can be further analyzed experimentally. The most important of these is the glucose transport kinetics in relation to the Tps1-mediated feedback of hexokinase. To estimate the potential error in the transport measurements, the intracellular glucose concentration should be measured. A low concentration of intracellular glucose would indicate correct glucose transport kinetics and therefore a requirement to reassess the (inhibition) kinetics of hexokinase. Alternatively, Tps1 might play only a minor role in this industrial strain. A high concentration of intracellular glucose (say 0.5 mM) would suggest that the true zero trans-influx kinetics were underestimated and that Tps1-mediated feedback of hexokinase should be included (to compensated for the increase in the glucose transport activity). The measurement of intracellular glucose may be accompanied by fast uptake kinetics over 200 ms (rather than 5 s), that should give a better estimate of the initial rate [343]. This is an excellent example of the heuristic power of modeling, as it pinpoints to the crucial experiment to discriminate between possible alternatives.

Product inhibition of aldolase should be measured under our conditions. The same should be done for PDC. Acetaldehyde inhibition of PDC may be studied by labeling of the methyl-carbon atom of pyruvate. This should allow the sensitive determination of pyruvate and the (newly formed) acetaldehyde, e.g. after HPLC separation. A way to determine the free concentration of cytosolic NADH may resolve the problems encountered for ADH and GAPDH.

The uncertainty in the rate equation for the general ATPase may be tackled by measurement of the ATP concentration at various glycolytic rates. When the fluxes through glycolysis and the branches are measured, the net rate of ATP production can be deduced. As in steady state the ATP production should equal the ATP consumption, the rate of ATP consumption can be calculated and compared to the steady state ATP concentration. This should result in a phenomenological rate equation for the ATP-consuming processes. The rate of glycolysis may be varied by modulating the extracellular glucose concentration. Addition of glucose at various rates (e.g. by a peristaltic pump) should allow a constant, low, glucose concentration in the medium. These experiments can also be used to arrive at phenomenological rate equations for the branch reactions, e.g. the glycogen production may be related to the G6P concentration.

Said experiments are also essential for more robust testing of the validity of our model. One steady state as used here will ultimately not be enough to validate it. Alternatively, conditions may be sought under which the cells exhibit glycolytic oscillations. Such dynamic states are even stronger tests to the model than steady states. Multiple steady states or dynamic
states also offer the possibility to discriminate which in vitro kinetic parameters require adjustment, $V_{\text{max}}$ values, equilibrium constants or affinity constants.

Another uncertainty that turned out to be extremely important, is the exact concentration of phosphate and the pH in the cytosol. Apart from their role in affecting the equilibrium concentrations in many reactions, they can affect the kinetics of many enzymes directly, such as those of PFK and PDC. These concentrations can and should be measured in vivo (although another buffer should be used).

Another way of testing the rate equations would be to measure fermentation in the cell free extracts under the same conditions as used for the enzyme kinetic determinations. In such a system, polyethylene glycol may be added to study the importance of protein-protein interactions on the system's performance. The advantage should be that unknowns associated with the exact cytosolic condition could be better controlled experimentally. Although we would like to understand glycolysis in intact yeast cells, such studies may help in specifying the uncertainties in the kinetics. A foreseeable problem with extracts is the lack of ATPases, but these may be mimicked by an ATP-hydrolase such as apyrase (see [387] for an excellent study in this direction).

The question addressed in the introduction, whether in vitro kinetics are able to describe in vivo behavior of a complicated metabolic pathway such as yeast glycolysis, is not completely answered. For half of the enzymes the in vitro kinetics did describe the in vivo activity satisfactorily, i.e. within a factor of two. For most of the enzymes that showed larger deviations, suggestions could be made that may have caused the discrepancy. In that way the analysis has played a guiding role for future experimentation that should lead to an improvement of our ability to understand the in vivo behavior of glycolysis on the basis of the properties of the glycolytic enzymes and their interactions.

**Acknowledgements**

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**8.6 Appendix I: kinetics of the enzymes.**

*Transport of glucose: HXT*

Glucose transport in yeast occurs via facilitated diffusion [106, 199, 213] and some 20 genes have been identified that form a family of putative hexose transporters [207]. Despite the large number of transporters, glucose transport in derepressed yeast cells can be kinetically
characterized by one high affinity component with a $K_m$ of 1-2 mM [383] (chapter 4). This was also the case for the yeast used in this study: zero trans influx of glucose occurred with a $K_m$ of 1.2 mM (Table II).

The way metabolism feeds back on the glucose transporter is a matter of debate. In the literature it is proposed that glucose 6-phosphate inhibits glucose transport [12, 306]. Such a feedback regulation has also been incorporated in most of the glycolytic models [77, 78, 108, 305, 324]. The experimental basis for this feedback is controversial [274]. We have shown experimentally that, for high-affinity transport, the simplest model for a facilitated diffusion transporter (a symmetrical carrier whose rate depends on extra- and intracellular glucose only), can fully account for the difference between initial uptake rates and steady-state glucose consumption (chapter 4, see also [343]). This mechanism has therefore also been used to describe the high-affinity glucose-transport rate for this yeast strain.

**Hexokinase: HK**

In yeast there are three enzymes able to phosphorylate glucose: hexokinase PI and PII, and glucokinase [222]. In our glucose-derepressed compressed yeast, hexokinase PI appears to be dominant: we found that $K_{m_{\text{fru}}}/K_{m_{\text{glu}}}=1.5$ and $V_{\text{max}_{\text{fru}}}/V_{\text{max}_{\text{glu}}}=2$ [222] (results not shown). This is in agreement with expression studies on the isoenzymes, which showed that hexokinase PII is predominant only at high glucose concentrations [148]. Our $K_m$ values for glucose and ATP agree well with published ones [17, 198, 222, 378]. The kinetics of hexokinase appear to be more complicated than the simple Michaelis-Menten kinetics employed in this study. For example, binding of glucose enhances the affinity for ATP [164, 378]. Such kinetics have not yet been incorporated. They may have implications for the role of glucose signaling by the hexokinases [83]. The impact of the more complex kinetics of hexokinase cannot be assessed yet, as data on the internal glucose concentration are yet lacking, and because product inhibition of the enzyme is not fully understood. Rather than being sensitive to its direct product, G6P (the $K_i$ was estimated to be 40 mM), hexokinase appears negatively regulated, in an as yet unknown way, by trehalose 6-phosphate synthase [363]. Competitive inhibition by Tps1's metabolic product trehalose 6-phosphate may offer a mechanism [38]. The existing models of yeast glycolysis do not take Tps1-mediated regulation of hexokinase into account, but rather, have incorporated G6P inhibition of glucose transport. In our model, also no Tps1-mediated feedback mechanism has been incorporated, as the feedback is not yet kinetically characterized (see main text for the consequences).

**Phosphoglucone isomerase: PGI**

PGI's kinetics have been measured in both directions and a reversible Michaelis-Menten equation was used to describe this enzyme. Our $K_m$'s are in agreement with those found in the
literature [409]. The kinetics fit reasonably well with the equilibrium constant \( K_{eq,P_{ALD}} = 0.31 \) according to the Haldane relationship: we found an equilibrium constant of 0.26.

**Aldolase: ALD**

Aldolase is generally assumed to follow an ordered uni-bi mechanism, with GAP binding after DHAP \([63, 303]\). The kinetic parameters are scarce, but all agree on the \( K_m \) for F16bP: around 0.3 mM \([17, 244, 302]\). Not many studies have been performed on the backward reaction, the only numbers found were from \([302]\). Unknown is the inhibitory constant for GAP: it was estimated by Richter \([303]\) and taken over by others (including us), but experimental data is lacking.

**Glyceraldehyde 3-phosphate dehydrogenase: GAPDH**

The enzyme GAPDH is an enigma. It is one of the most abundant enzymes in yeast \([139]\). In *S. cerevisiae*, it has been found in the cytosol and in the nucleus, and in some related yeast species it appears also located at the cell wall \([130]\). The enzyme appears to bind specific tRNA's \([336]\).

The kinetics of the enzyme are renowned for their complexity. The enzyme forms monomers, dimers and tetramers which have different activities \([269]\). Many factors may affect the distribution of oligomers, most notably NAD and NADH \([320]\). Cooperative binding of NAD and NADH has been well documented \([58, 93, 139]\). These binding properties could be described by a concerted model of the Monod, Wyman, Changeux type \([93]\). However, the cooperativity is only clearly seen at high pH, pH>7.5 \([58]\). A complete data set of \( K_m \)'s under very similar conditions as ours was found in the literature \([211]\); they also did not observe cooperativity. We have therefore not included cooperative binding in the rate equation, but rather used reversible two-substrates, two-products Michaelis-Menten kinetics. We have measured the \( V_{max} \) in both directions. Inhibition by the adenine nucleotides has been reported \([410]\) and incorporated in some models \([77, 108]\), but their effects were very small with the inhibition constants and concentrations of the adenine nucleotides used in the model (result not shown). Therefore this inhibition has been left out.

Using the Haldane relationship, the calculated equilibrium constant, using our maximal rates and the affinities of Lambeir \([211]\), differed 5-fold with the published equilibrium constant \([59]\). The most suspect parameter in the rate equation of GAPDH is the affinity for 13PGA. 13PGA was varied indirectly by in situ generation of 13PGA by PGK, using excess 3PGA and variable amounts of ATP. It was assumed that the ATP concentration added equaled the 13PGA concentration at the excess of auxiliary enzyme added \([211]\). We have changed the uncertain \( K_m \) of 13PGA (5-fold) to arrive at the right equilibrium constant for GAPDH.

**Phosphoglycerate kinase: PGK**
PGK is difficult to assay in the forward direction because of the instability of its substrate, 13PGA. Many studies have been performed on the reverse reaction, however, with $K_m$ values for 3PGA in the range 0.2-0.5 mM [17, 236, 312, 325, 333], and for ATP between 0.1 and 0.5 mM [17, 206, 236, 325, 333]. Our values of 0.53 mM for 3PGA and 0.3 mM for ATP agree with those. The $K_m$ for 13PGA was estimated by the $K_i$ as measured by [322]. The $K_m$ for ADP was from [206].

**Phosphoglycerate mutase: PGM**

PGM in yeast is dependent on 2,3-diphosphoglycerate for activity. We have not taken 23PGA into account, assuming that the enzyme is saturated for 23PGA. The $K_m$ for 23PGA is in the low micromolar range [132], whereas the concentration of 23PGA was estimated to be 0.1 mM (result not shown). The affinity of PGM for its substrate 3PGA differs quite a lot between studies. Values from 2 mM to 0.2 mM have been reported [17, 115, 132, 399]. We found a value of 1.2 mM. For the product 2PGA, a value of 0.08 mM was used [115].

**Enolase: ENO**

Two isoenzymes of enolase are present in yeast. Class I enolase is expressed under glucose derepressed conditions; class II enolase is expressed during growth on glucose [94]. We found a $K_m$ value for 2PGA that agrees well with that found for class I enolase [94], in line with the derepressed state of the compressed yeast. Subsequently the $K_m$ of the product PEP was taken from class I enolase [94].

**Pyruvate kinase: PYK**

The most striking feature of pyruvate kinase is its strong activation by F16bP. At low F16bP concentrations prevailing during gluconeogenic conditions, the enzyme showed cooperativity with respect to PEP. At high F16bP concentrations, however, the enzyme exhibited hyperbolic Michaelis-Menten kinetics with increased affinity for PEP, in line with earlier findings [154, 250]. The regulation by F16bP is mostly likely involved in the transition between glycolytic and gluconeogenic conditions. We found that the activation is maximal at 0.5 mM of F16bP, which is more than ten times lower than the concentration found under our conditions. We have therefore used Michaelis-Menten kinetics. Our $K_m$ values for PEP and ADP fit well with the values found in numerous studies [17, 19, 154, 171, 176, 237, 250, 400, 414]. The affinities of the products are much less studied: we have estimated them from the dissociation constants measured by Macfarlane [228].

**Pyruvate decarboxylase: PDC**

Pyruvate decarboxylase shows cooperative kinetics with respect to its substrate pyruvate [41, 170]. The cooperativity and affinity of pyruvate are phosphate dependent [41, 170]. We have adopted the $K_m$ of 4.3 mM and Hill coefficient of 1.9 as measured by Boitelux [41] at 25 mM.
phosphate. This $K_{0.5}$ value is in line with the 4 mM found by [17] (see also [281] and references therein).

**Alcohol dehydrogenase: ADH**

Of the 5 isoenzymes of ADH present in yeast, two have clearly established metabolic functions: ADHI is glucose induced and is therefore present during glucose fermentation; ADHII is glucose repressed and predominant under derepressed conditions. We have estimated the contribution of ADHI and ADHII to the total ADH activity by varying the ethanol concentration and measuring the initial rate of NAD reduction in cell-free extracts. As the two enzymes differ in their affinity for ethanol by one order of magnitude (ADHII having the higher affinity [112]), the two components were expected to be visible in this way. Indeed, Eadie-Hofstee plots of ADH activity versus ethanol showed curved kinetics, indicative of more than one component (cf. use of Eadie-Hofstee plots for this purpose in glucose transport kinetics [73, 383]). When two components were fitted, $K_m$ values of 45 ± 10 and 0.7 ± 2 mM were found, with corresponding $V_{max}$ values of 2.8 ± 0.2 and 0.3 ± 0.2 U mg total protein$^{-1}$. Although the errors are substantial, especially in the high affinity component (corresponding to ADHII), it can be concluded that the low-affinity component (corresponding to ADHI) is the dominant isoenzyme present under our conditions. It was checked that also the in vitro activity of ADHI was tenfold higher than that of ADHII when the measured metabolite levels (Table I) were substituted in the rate equation for ADH. We have therefore used the kinetics of ADHI only.

The reaction of ADH follows ordered bi-bi kinetics [112]. Kinetic parameters can be found in [112, 402, 406]. We have chosen the data from Ganzhorn, as for each isoenzyme a complete data set including inhibition constants was given, under conditions very similar to ours [112]. The affinities for ethanol found by us are in reasonable agreement with the ones reported by Ganzhorn (17 and 0.8 mM for ADHI and ADHII, respectively).

**Glycerol 3-phosphate dehydrogenase: G3PDH**

G3PDH has not been extensively studied as far as the kinetics are concerned. It is important in regulating glycerol production during osmotic stress and is likely to have significant control over the glycerol flux [39]. We have measured the affinity of G3PDH for DHAP, which was in agreement with that measured by Albertyn [6]. Kinetic constants for NAD and NADH were from [6]. No data were found on the affinity of G3PDH to glycerol 3-phosphate. We have adopted a value of 1 mM. The limiting rate of G3PDH turned out to be very sensitive to the salt and protein concentration in the assay, and no reliable $V_{max}$ could be measured. Instead, we have adjusted G3PDH's $V_{max}$ to the measured glycerol flux.
8.7 Appendix II: Kinetics of phosphofructokinase

PFK may be an enzymologist’s favorite, it is a modeler’s nightmare. The difficulty is the many regulatory interactions and the resulting combinatorial explosion. Simplification is therefore required, and many effectors are necessarily assumed to be constant in the time window of the model, such as ammonium, phosphate, protons and fructose 2,6-bisphosphate. The regulatory effects that have been used explicitly in the existing glycolytic models are the cooperative binding of F6P, the inhibition by ATP (in some), the activation by AMP (in all [77, 78, 108, 303, 305, 324]) and ADP (only Rizzi [305]). The role of ADP in regulation of PFK is much less important than the ones of ATP and AMP, and has not been included in our rate equation for PFK.

Product inhibition by F16bP is not included in any of the existing models. Our analysis of the consequences of the autocatalytic stoichiometry of glycolysis showed that such a product sensitivity has important consequences for the flux control distribution (chapter 7), and has to be included. The main inhibitory action of F16bP is a decrease in the activation by F26bP [18, 268, 282]. Therefore, a minimal kinetic model of PFK should be a function of the concentrations of F6P, ATP, AMP, F26bP and F16bP. We have measured the activity of PFK in partially purified

Figure 4. Kinetics of PFK as a function of its substrates ATP and F6P. A: PFK activity as a function of F6P at different concentrations of ATP; 0.0125 mM (●), 0.5 mM (△), 2 mM (○) and 5 mM (□). B: PFK activity as a function of ATP at different F6P concentrations; 0.1 mM (●), 0.6 mM (△), 1 mM (○) and 3 mM (○). The lines indicate the fits through the data points.
Figure 5. AMP activation of PFK at a concentration of ATP of 1 mM. AMP was varied between 0.025 mM and 5 mM. The lines indicate the fits through the data points.

Figure 6. F26bP activation of PFK at a concentration of ATP of 1 mM. F26bP was varied between 0 mM and 0.02 mM. The lines indicate the fits through the data points.

enzyme preparations (see Methods) as a function of F6P and ATP, and then looked at the effects of AMP and F26bP. The inhibitory effect of F16bP was incorporated on the basis of data from Otto [268]. To our knowledge, no model is available that describes all these effects at the same time. We have successfully tried to fit the same model as was used by Galazzo [108], Schlosser [324] and Cortassa [77]. It is based on the Monod, Wyman, Changeux model for allosteric enzymes, adapted by Hess [155] to two substrates.

The effect of AMP, F26bP and F16bP, and the inhibitory effect of ATP are assumed to be mediated by displacement of the equilibrium between the Tense state and the Relaxed state, i.e. they effect the equilibrium constant L. It was assumed that the Tense state did not bind F6P and thus, that this state is inactive. The rate equation that was used to fit the data was therefore:

\[ V_{PFK} = V + \frac{\frac{g_R \lambda_1 \lambda_2 R}{R^2 + LT^2}} \]

with:

\[ \lambda_1 = \frac{[F6P]}{K_{R,F6P}} \]  
\[ \lambda_2 = \frac{[ATP]}{K_{R,ATP}} \]  

and:

\[ R = 1 + \lambda_1 + \lambda_2 + g_R \lambda_1 \lambda_2 \]  
\[ T = 1 + c_{ATP} \lambda_2 \]  

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\[ L = L_0 \left( \frac{1 + c_{i,\text{ATP}} [\text{ATP}]}{1 + [\text{ATP}]/K_{\text{ATP}}} \right)^2 \left( \frac{1 + c_{i,\text{AMP}} [\text{AMP}]}{1 + [\text{AMP}]/K_{\text{AMP}}} \right)^2 \]

\[ \left( \frac{1 + c_{i,\text{F26bP}} [\text{F26bP}]}{K_{F26bP} + [\text{F26bP}]/K_{F16bP}} \right)^2 \left( \frac{1 + c_{i,\text{F16bP}} [\text{F16bP}]}{K_{F16bP} + [\text{F16bP}]/K_{F26bP}} \right) \]

The kinetic parameters for F6P and ATP were estimated by non-linear regression of over 200 data points of the rate of PFK as a function of the concentrations of ATP and F6P (see Fig. 4 for a selection of data and the fit through them). Kinetic parameters are shown in Table II. They are quite different from the ones used in the other models, most notably \( L_0 \) [108]. One reason may be that ATP inhibition was explicit in our equation, where it is implicit (and ATP independent) in the \( L_0 \) of the equation used by the other groups. The ATP inhibition factor increases \( L \) by at least three orders of magnitude at physiological ATP and F6P concentrations. Hofmann's group, however, have also found low \( L_0 \) values [35, 187, 260], in good agreement with ours.

Activation of AMP was measured at different F6P concentrations, with an ATP concentration of 1 mM (see Fig. 5). These data were used to fit the binding parameters of AMP for the Tense and Relaxed state. The same procedure was followed for F26bP activation (Fig. 6). F16bP decreases the activation of F26bP, most probably by competition for the binding site. This can explain activation of PFK by F16bP in the absence of F26bP (results not shown, see [18, 98]). Otto showed an inhibition of F26bP activation by F16bP. His data was used to fit a model describing competition of F16bP and F26bP for the same site (fig. 7). The same mechanism was used by Kessler et al to describe the inhibitory effect of F16bP on the activation by F26bP [186]. A model of inhibition by F16bP via competition with F6P

Figure 7. F16bP inhibition of F26bP activation of PFK. A: ATP = 3 mM, F26bP = 0 mM and F16bP = 0 mM (●), ATP = 3 mM, F26bP = 0.02 mM and F16bP = 10 mM (○), ATP = 3 mM and F26bP = 0.02 mM (●). B: ATP = 3 mM, F6P = 0.3 mM and F26bP = 0.02 mM (O), ATP = 0.3 mM, F6P = 0.3 mM and F26bP = 0.02 mM (●). The lines indicate the fits through the data points.
could not describe the observed inhibition curves (data not shown).

To our knowledge this is a first attempt to capture the effects of so many metabolites in a single rate equation. Our rate equation was able to satisfactorily describe the effects of the substrates F6P and ATP, the inhibition by ATP, the activation by AMP and F26bP and the inhibitory effect of F16bP on the activation by the latter.
The kinetic parameters for PEP and ATP were estimated by nonlinear regression of over 200 data points of the rate of PK as a function of the concentrations of ATP and PEP (see Fig. 4) for a selection of data and the fit through the model. Kinetic parameters are shown in Table 1. They are quite different from those used in the other models, most notably L1069. One reason may be that ATP inhibition was explicit in our equation, whereas it is implicit (and ATP independent) in the 1 of the equation used by the other groups. The ATP concentration factor increases L by at least three orders of magnitude at physiological ATP and GTP concentrations. Holzmann's group, however, have also found low L values [105, 187, 200], in good agreement with ours.

Activation of AMP was measured at different NADP concentrations, with an ATP concentration of 100 μM (see Fig. 5). These data were used to fit the binding parameters of AMP to the sense and closed state. The parameters were obtained for L, 0.49, 0.91, and 0.53 (see Fig. 5). Fluctuation decreases the activation of 20/24 units, which cannot be explained by competition for the binding site. This can explain the behavior of 0.49 in (700) or 0.45 in (700) results not shown in Fig. 5, but not enough of inhibition of 0.91. Therefore, the data were used in fit a different curve to obtain 0.49 and 0.91. In conclusion, the curve fitting data was in good agreement with the model.