Exposing a complex metabolic system: glycolysis in Saccharomyces cerevisiae

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CHAPTER 9

GENERAL DISCUSSION AND PERSPECTIVES

9.1 Summary:

In this thesis, the systemic properties of yeast glycolysis have been studied starting from the properties of individual enzymes and their interactions. Throughout the thesis, experimental work was compared with mathematical models, and vice versa. This combined approach has led to deeper insight in how yeast glycolysis is controlled and regulated. In this final chapter, I will highlight some of the results that led to better understanding of metabolic pathways, yeast glycolysis in particular, by bringing results of different chapters together and putting them into perspective. I will also use this chapter to ventilate some ideas I have accumulated in the last few years and discuss problems that are still waiting to be explored. I will suggest experiments for which I unfortunately have not found time, in the hope that they will stimulate other people to prove me right or wrong.
9.2 Oscillations and turbo design

Chapters 2 and 3 and part of chapter 5 dealt with a fascinating systemic behavior: glycolytic oscillations. Periodic behavior is quite a significant feature of biological systems [124]. Glycolytic oscillations are a readily accessible model system to study how such oscillations can be controlled, especially since the experimental conditions for reproducible sustained oscillations have been established [300]. Additionally, intact yeast cells show mutual synchronization. The compound that mediates this intercellular coupling has been identified [297]. The extent of cell-cell coupling can therefore be modulated experimentally and oscillating yeast cell populations are therefore also very interesting as an experimental system to test theoretical studies of coupling of oscillators [405].

The question of what controls the characteristics of glycolytic oscillations, has been confused by what intuitively seemed a nice metaphor: the oscillophore PFK as a pump of the glycolytic system. Although the importance of PFK in the generation of oscillations is undisputed, the dismissal of the term oscillophore in chapter 2 is not merely a semantic argument. The image of PFK as a pump is readily taken to imply that PFK therefore controls the characteristics of the oscillation. Although this implication has not been explicitly stated by Hess and colleagues, it appeared to be adopted by others. Using Metabolic Control Analysis we have shown, by computer modeling in chapter 2 and experimentally in chapter 5, that the frequency of glycolytic oscillations is a systemic property controlled by all enzymes, not just by the oscillophore.

This conclusion should be applicable to any other oscillating biological system. In most models explaining oscillatory phenomena, positive feedback (or negative feedback with delay) plays an important role. Thus, mechanisms for Ca²⁺-oscillations include Ca²⁺-induced Ca²⁺-release, or Ca²⁺-stimulation of inositol 1,4,5-trisphosphate generation (which stimulates Ca²⁺ release from the intracellular stores). Similarly, the oscillation of cAMP in and around Dictyostelium discoideum, involved in the process of aggregation of the unicellular amoeba, is explained by activation of intracellular cAMP synthesis by extracellular cAMP (see [124] and references therein). Such mechanisms may point at some specific steps being essential for the oscillations to occur, similar to PFK in the case of glycolytic oscillations. Although such steps are important, they do not a priori have more influence on the frequency or amplitude of the oscillations than does any other step of the system.

The involvement of positive feedback in the mechanism of oscillations appears related to the danger of glycolysis' design discussed in chapters 6 and 7. There it was shown that the autocatalytic stoichiometry (turbo design) of glycolysis is responsible for a deleterious overflow effect caused by removal of the feedback on hexokinase. The same autocatalytic stoichiometry of glycolysis has been shown to be able to generate oscillations [79, 334]. How can the same
design of glycolysis lead to these two phenomena? Whether the design leads to overflow or oscillatory behavior depends on whether there is a limitation in the ATP producing steps or not. If there is such a limitation, the ATP concentration will decrease until it reaches a state at which ATP consumption and ATP production are equal, whereas the rate of the upper part of glycolysis is still higher than that of the lower part of glycolysis (chapter 6). Accumulation of intermediates, most notably F16bP, occurs. Because of the limitation in the ATP producing steps, the accumulation of F16bP does not give rise to increased ATP production, and no steady state is reached, or a steady state with extremely high levels of F16bP.

If there is no limitation in the ATP-producing step, however, accumulation of F16bP does lead to enhanced ATP production in the lower part of glycolysis. A stable steady state may be reached (as shown in chapter 6 for the reduced HK model). This steady state may also be unstable, however, depending on the kinetics of the steps involved. As the stoichiometry is autocatalytic, a 1% increase in the rate of the lower part of glycolysis can give a 2% higher rate of ATP production. This ATP may boost the upper part of glycolysis to form more F16bP, which would lead again to even more enhanced ATP-production. The basis for the instability (and hence, the possibility of occurrence of oscillations) is therefore that an increase in F16bP, via the autocatalytic link of ATP, can stimulate the production of even more F16bP. The more stimulated the ATP-consuming steps producing F16bP are to increased levels of ATP, the more likely the instability of the steady state.

The design of glycolysis is therefore prone to instabilities and to overflow. Yet, the turbo design of glycolysis appears optimized to the rate of ATP production [142]. For the danger of overflow, the disadvantage is obvious and mechanisms have arisen to prevent this. How about oscillations, then? Are oscillations an accident occurring under very specific conditions, or may there be some functional advantage to glycolytic oscillations? Whether glycolytic oscillations only occur under very specific conditions, remains to be established. The specific conditions employed in our experiments, may be required to keep yeast cells in phase and hence, to observe the oscillations macroscopically. We have some indications that this may be the case. Titrations with pyrazole, an inhibitor of ADHII, could increase the duration of the oscillations of cells harvested 3 h after diauxic shift [Teusink and Veldkamp, unpublished results]. Normally, these cells show shorter oscillations than cells harvested at diauxic shift (chapter 3). This result may be rationalized by the involvement of ADHII in the synchronization of oscillations via acetaldehyde. ADHII has a much higher affinity for acetaldehyde than ADHI, and a critical activity of ADHII may be required for proper coupling of the cells through acetaldehyde. The strong damping of oscillations in cells harvested during exponential growth on glucose may then be explained by the absence of ADHII, which is glucose repressed. Experiments with a yeast strain constitutive in ADHII should reveal whether indeed the growth-state dependence of the damping of glycolytic oscillations does derive from the dependence on a critical expression level of ADHII. Alternatively, single cell oscillations may be monitored by fluorescence microscopy of
NADH. This should discriminate between all cells not oscillating and all cells oscillating but out of phase.

The possibility that the specific experimental conditions employed to observe macroscopic oscillations are merely required for proper synchronization of the cells, may have profound effects on the study of yeast glycolysis. Its consequence may be that steady states observed on the macroscopic level (such as in chapter 8) in fact reflect an average of many asynchronously oscillating systems, rather than a true steady state. This may then also occur during dough leavening and beer brewing. Inhomogeneity caused by improper mixing of large volumes may also contribute to a highly dynamic glycolytic system. The relation between the dynamics of the glycolytic pathway and variation in the extracellular milieu are the subject of further study by Karin Reijenga in the laboratory of Hans Westerhoff and Jacky Snoep.

Is it possible to assign a function to glycolytic oscillations? This has been proven to be rather difficult. One suggestion has been made on the basis of theoretical studies on the efficiency of glycolysis during oscillations and during steady state, evaluated at the same (average) flux [304]. The conclusion was that the efficiency of glycolysis could be 10% higher during oscillations than during steady state. This conclusion is dependent on the definition of efficiency, however. Moreover, it is unlikely that this is a good selection criterium, since glycolytic oscillations occur during fermentation of relatively high glucose concentrations, where rate, rather than efficiency appears important (cf. [391]).

Other suggestions are similarly doubtful. An increased average ATP/ADP ratio was suggested for skeletal muscle extracts [365]. Oscillations have been suggested to prevent futile cycling [43]. An oscillating energy charge was modeled [123], and experimentally observed [299]. This might allow processes to occur at the maximum of the energy charge that would not be possible at a steady state with the average energy charge.

An alternative would be that oscillations are involved in signaling, similar to frequency-encoded hormone signals and firing of neurons [289, 290]. According to this hypothesis, the frequency of the glycolytic oscillations is to a large extent controlled by the concentration of extracellular glucose (or better, the activity of the transporter). The frequency of the oscillations (e.g. in the internal glucose concentration) could then constitute a signal reflecting the extracellular glucose concentration.

More down-to earth suggestions for the function of glycolytic oscillations come from recent observations that glycolytic oscillations may underlie the pulsatile secretion of insulin [70], or Ca²⁺-oscillations in heart muscle [267]. Thus, glycolytic oscillations in yeast may be an accident that is not detrimental to the cell (or may not occur outside the laboratory), but the potential of glycolysis to oscillate has been used by higher eucaryotes. Even if the glycolytic oscillations are a laboratory artifact without any advantage to the yeast cells, they have proven to be useful as a prime example of biochemical oscillations. Much of the insight in how oscillations in biology may occur, has originated from the study of yeast glycolytic oscillations.
Moreover, they should be an excellent validation system for future improvements on the model described in chapter 8.

9.3 Control and regulation in glycolysis: the role of hexokinase and phosphofructokinase

A longstanding and important question addressed in this thesis is to what extent the enzymes in glycolysis control the glycolytic flux. Experiments where the glycolytic enzymes were overexpressed, suggested that flux control is not in the glycolytic enzymes themselves [319]. Consequently, control can reside in the glucose transport step and steps outside glycolysis, such as biosynthetic pathways and (other) ATP-consuming processes. The role of glucose transport will be discussed in the next section.

Glycolysis serves the function of producing free-energy in the form of ATP, as well as precursors for biosynthesis. Glycolysis may therefore be seen as what has been called a supply pathway [160]. As discussed by Hofmeyr and colleagues [159, 160], a well-regulated metabolic system should put control in the demand, not in the supply, i.e. the supply reactions should only produce products when there is a demand for it (see also [396]). Hofmeyr and colleagues focused their analysis on amino acid biosynthesis. For amino acid biosynthesis, the supply block would be the reactions forming an amino acid, whereas the demand block comprises the reactions utilizing the amino acid (i.e. protein synthesis). If the substrate of the supply block is assumed to be abundant, the kinetic properties of the enzymes in many amino acid biosynthesis pathways, such as the well-known feedback inhibition by the amino acid on the first step of the biosynthetic route, could be elegantly explained by postulating control by demand [98, 160].

For yeast glycolysis, a similar situation may arise. If the substrate for glycolysis is abundant, following the reasoning of Hofmeyr and coworkers, the ATP-consuming processes and biosynthetic processes should control the rate of glycolysis. The glycolytic enzymes and probably also the glucose transporter should not exert much control.

If it is true that in yeast control of the glycolytic flux resides outside glycolysis when yeast cells are happily growing on a high concentration of glucose, how do the controlling steps act on glycolysis? The question is how the glycolytic flux is regulated (not controlled). For this, we should take glycolysis in isolation and treat the signals from the demand steps as external effectors. To emphasize the difference between the glycolytic flux in isolation and that flux embedded in the rest of metabolism, the former flux will be called supply flux, \( J_s \).

The importance of an enzyme in the regulation by any of these external effectors is quantified by the regulatory strength of an enzyme with respect to that external effector. The regulatory strength of an enzyme is a partial response coefficient, i.e. it is the relative change in a metabolic variable upon a change in a parameter (the external effector), effected by that
The regulatory strength of enzyme \( i \) in the regulation of the supply flux \( J_s \) by effector \( X \) is therefore:

\[
R_{X}^{J_s} = C_{i}^{J_s} \cdot \varepsilon_{X}^{i} \tag{9.1}
\]

The total response of the flux to effector \( X \) is the sum of the partial response coefficients of all enzymes. The regulatory strength of an enzyme in the regulation of the flux by effector \( X \) is determined by the elasticity of that enzyme to the effector (its “regulability”) and by the control exerted by the enzyme on the flux (also called “regulatory capacity” [160]). An enzyme that has a high control on the flux, but is insensitive to a particular effector, will have only a small regulatory strength. An enzyme that is very sensitive to the effector, but has no control on the flux, will also be regulatory “weak”.

In yeast glycolysis, PFK is the enzyme most sensitive to all sorts of metabolic signals. For these signals to be effective, PFK should control the supply flux to a significant extent (i.e., the glycolytic flux in isolation from the rest of metabolism). Can we understand the regulatory mechanisms in glycolysis in the light of PFK requiring high control on the supply flux? For one, the saturation of the F16bP inhibition of PFK at the high steady-state concentration of F16bP prevailing at high glucose concentrations (chapter 8), ensures that control of the supply flux is mainly in PFK and upstream reactions (chapter 7).

For simplicity, it is assumed that the PGI reaction does not have significant control on the supply flux. Glucose transport is also not yet considered, as I would like to focus on the competition for control between HK and PFK. With PGI in equilibrium and taking \( G6P + F6P = HMP \) (hexose monophosphates; chapter 6), the flux control coefficients of these two enzymes read:

\[
C_{H,K}^{J_s} = \frac{1}{1 - \varepsilon_{H,K}^{HMP} / \varepsilon_{H,K}^{PFK}} \tag{9.2a}
\]

\[
C_{PFK}^{J_s} = \frac{1}{1 - \varepsilon_{PFK}^{HMP} / \varepsilon_{PFK}^{HMP}} \tag{9.2b}
\]

Thus, the control distribution between these two kinases depends on the relative elasticities of the enzymes for the linking pool, HMP. If HK were strongly inhibited by its product (i.e. if \( \varepsilon_{H,K}^{HMP} \) were high), control by HK should be small: feedback inhibition shifts control from the inhibited enzyme (HK) to the enzyme consuming the inhibitor (PFK). This should increase the regulatory capacity and hence, the regulatory strength of PFK. The feedback inhibition of HK by G6P found in most mammalian cell types may have this function. It is notable that glucokinase in insulinoma cells of the Islets of Langerhans is not sensitive to G6P, which confers all control
on the glycolytic rate to this enzyme (and the glucose transporter, but that step has a very high capacity in beta cells [385]). This fits with the role of glucokinase in these cells in sensing of the blood glucose level, in response of which the cells produce insulin.

In yeast, Tps1 may have substituted for G6P-inhibition. The need for a regulatory loop rather than direct product inhibition in yeast was proposed in chapter 6 to arise from the large variation in external glucose concentrations encountered by yeast. A regulatory mechanism such as Tps1 may increase the dynamic range of the inhibition. It may also lead to a much higher effective $v_{H_K}^{EHP}$ than can be attained with product inhibition.

The product insensitivity of PFK and the Tps1-mediated feedback inhibition on HK may serve to ensure that PFK has a high regulatory strength. PFK can then adjust the glycolytic rate when it is told to do so. A necessary supplement to the textbooks is, therefore, that PFK does not so much control the glycolytic flux, but that it has a high regulatory strength. For the latter, it should have a high control on the supply flux, i.e. the glycolytic flux in isolation, but not on the glycolytic flux in the intact cell. Consequently, overexpression of PFK does not increase the glycolytic flux [81, 319], as glycolysis is embedded in the rest of metabolism.

Is it possible to explain the function of F26bP regulation of PFK in yeast within the same concept of PFK having high regulatory strength but limited control? A strain deleted in the genes encoding for PFK-2 (i.e. PFK26 and PFK27) and hence, without any F26bP, showed a 3-fold increase in the level of F6P as compared to the wild type [45]. This increase presumably serves to counteract the absence of the positive effector. The effect of such an increase in F6P on the ability of the adenine nucleotides to regulate PFK is shown in Fig. 1. In Fig. 1A the rate of PFK is calculated as a function of the energy charge (defined as [ATP+0.5ADP]/[ATP+ADP+AMP]), using the kinetics of PFK described in chapter 8. Adenylate kinase was assumed to be in equilibrium. For the F26bP-negative mutant, the F6P concentration was taken 3 times higher than in the wild type. Inhibition of PFK by the energy charge is less effective in the mutant than in the wild type. This is clearly demonstrated in Fig. 1B, where the elasticity of the PFK reaction towards the energy charge is shown for the mutant (no F26bP present) and the wild type (0.02 mM F26bP present). In the wild type, the presence of F26bP (and hence, a low F6P concentration) led to two- to eightfold enhanced sensitivity of PFK to the energy charge as compared to the mutant without F26bP.

Regulators of PFK that act on the distribution between the Tense and Relaxed state of the enzyme (chapter 8), will be less effective at high concentrations of F6P than at low concentrations of that substrate (as shown here for the adenine nucleotides). The primary role of F26bP may therefore be to reduce the concentration of F6P and thereby enhance the regulability (elasticity) of PFK for regulators of the enzyme. This would increase the regulatory strength of PFK over the supply flux.
From Eq. 9.2 it is evident that HK should be more elastic to HMP than is PFK when PFK is supposed to exert more control on the supply flux than HK. Changes in the level of HMP may affect the elasticity of these enzymes for HMP and hence, the control distribution between these two enzymes. A decrease in HMP should lead to a decreased elasticity of HK for HMP, but it also tends to decrease the elasticity of PFK for HMP (result not shown). There is not enough (quantitative) data available to predict the net result on the control distribution between HK and PFK. The (strong) positive effect of F26bP on the regulability of PFK (i.e. its elasticity towards regulators), however, does not depend on how $\varepsilon_{HMP}^{HK}$ and $\varepsilon_{PFK}^{HK}$ are affected by the concentration of HMP. I therefore suggest that the role of F26bP is to increase the regulatory strength of PFK via a decrease in the concentration of F6P.

Figure 1. Effect of the energy charge on the rate of PFK (A) and on the elasticity of PFK for the energy charge (B). The rate of PFK was modeled using the rate equation described in chapter 8, with F16bP = 5.5 mM and F26bP = 0 (pfk26A pfk27A mutant) or 0.02 mM (wild type). For the wild type, a F6P concentration of 0.61 mM was used. For the mutant, the concentration of F6P was increased by a factor of 3.

The role of F26bP in keeping the concentration of F6P low, may also explain the observed longer lag-phase after transfer from ethanol to glucose medium in mutant strains lacking any F26bP [45]. The high concentrations of G6P and F6P may enhance the Tps1-mediated inhibition of HK more strongly than required to tackle the 'turbo' problem. The stronger inhibition may then reduce the consumption of glucose. Moreover, high levels of G6P and F6P may lead to higher fluxes through the glycogen and trehalose branches. Glucose consumption appears to be less affected by the gene deletions than ethanol production, i.e. shows a shorter lag phase after transition from ethanol medium to glucose medium [44]. Long term effects such as glucose repression of Tps1 and glycogen synthase may relieve inhibition on HK and decrease the flux.
into storage carbohydrates, so that the glycolytic flux reaches wild type level. Measurement of the levels of these storage compounds during a transition from ethanol to glucose would be required to test these ideas.

It would also be interesting to analyze a hyp/pfk26A/pfk27A mutant (i.e. a mutant lacking F26bP and having only 10% of the wild type level of Tps1 activity). Such a strain may allow accumulation of hexose monophosphates to accommodate the HK and PFK activities, without PFK becoming too active for the lower part of glycolysis (chapter 7). As the outcome is very much dependent on a subtle balance between inhibition of HK and activation of PFK, controlled expression of TPS1 may be required (by putting the gene behind one of the promoters discussed in chapter 1). According to the ideas presented above, a lower expression level of TPS1 for growth on glucose may suffice in the absence of F26bP than in its presence.

To test whether F26bP is required for proper regulation of glycolysis by transferring more regulatory strength to PFK, the growth of a pfk26Apfk27A mutant may be compared with that of a wild type strain under variable external conditions, e.g. by varying the ammonium concentration in the medium, by aerobic/anaerobic transitions and/or by adding uncouplers such as benzoic acid. The mutant should then have more difficulty to respond adequately to the changing environment.

9.4 Flux control by the hexose transporter and its consequences for the transport kinetics

So far the control exerted by the glucose transport step on the glycolytic flux has not been considered. If the analysis of Hofmeyr and coworkers is followed again, glucose transport is part of the supply block and should have very little control when cells are growing on high concentrations of glucose. The situation may be more complicated, however, as transport of glucose is also important for glucose signaling. The role of hexose transport in signaling may affect the proportion of glucose being respired or fermented, however. Fermentation of glucose into ethanol is much less efficient in terms of ATP formation than is “respiration of glucose”, and a higher glycolytic flux would be required to sustain the same growth rate in the former case. Whether glucose transport controls the growth rate is therefore a different question from whether glucose transport controls the glycolytic flux.

There is some evidence that suggests that sugar transport does not have a high control on the growth rate at high glucose concentrations, but may have significant control at low concentrations. In E. coli, control of the growth rate by the IIICB component of the PTS glucose transport system was zero during batch growth [313], whereas it was significant during continuous cultivation under glucose-limitation [412]. Although in continuous cultivation the growth rate is completely controlled by the dilution rate, control over growth rate can be
assessed in a chemostat via the effect on the residual substrate concentration [345]. The growth control coefficient thus found corresponds to the growth control coefficient that would have been found if the cells were to be freely growing at the low glucose concentration (e.g. in a substratostat in which not the growth rate but the extracellular glucose level were controlled).

There is indirect evidence that, also in yeast, control over growth rate resides (to a significant extent) in glucose transport when cultivated in glucose-limited chemostats. If glucose transport is limiting growth to any extent, this should make the glucose transporter a target for selection, especially in yeast where there is a large family of hexose transporters. This has indeed been observed in a glucose-limited continuous cultivation of wild type yeast over a very long period of time. During the cultivation mutant strains were selected with multiple copies of the genes encoding the high affinity transporter [53]. The increased transport capacity cannot lead to an increased growth rate (which is controlled by the dilution rate), but it should decrease the residual glucose concentration, which then results in a slower growth rate of the non-mutated strains and their consequent washout.

A similar phenomenon was observed when a mutant strain deleted for all physiologically relevant $HXT$ genes, except $HXT1$ (a low affinity carrier), was grown in a glucose-limited chemostat. Suppressor mutations introducing high-affinity glucose transport were selected for, possibly by mutations in the $GAL$ signaling cascade. Such mutations led to constitutive expression of $GAL2$, the galactose permease, in the absence of galactose. $GAL2$ transports glucose with high affinity [Schepper, personal communication].

These examples suggest that in continuous cultures at low glucose concentrations, control of the growth rate does reside to a significant extent in glucose transport. How about growth on high glucose concentrations? During growth on high glucose concentrations, low-affinity carriers are expressed. One interpretation of this would be that as soon as the glucose concentration increases, the affinity of the carrier decreases to ensure that the hexose carrier retains significant control over the growth rate. There is some evidence, however, that glucose transport has little control over growth rate during high glucose cultivation in yeast, similar to what was found for $E. coli$. Walsh et al [382] showed that a mutant deleted in $HXT1,3$ and $4$ grew equally fast on 2% glucose as did the isogenic wild type, whereas its maximal rate of glucose transport and its affinity for glucose were (surprisingly) increased by a factor two. Moreover, introduction of a plasmid harboring a sugar permease from the filamentous fungus $Trichoderma reesei$ into a wild type $S. cerevisiae$ strain, did not lead to an increased growth rate on glucose [Ruohonen and Walsh, unpublished results]. The introduction of the permease into the null strain (a strain with all physiological $HXT$ genes deleted, see chapter 1) did restore growth on glucose, demonstrating that this permease is expressed and functional in $S. cerevisiae$. These results demonstrate that introducing more transporters into a wild-type yeast, most likely will not result in an increased growth rate.
As said in the beginning of this section, the glycolytic flux is not necessarily related to the growth rate. Indeed, in the experiments described above, effects were seen on the hexose consumption rate even though the growth rates remained the same. To what extent hexose transport exerts control over the glycolytic flux during growth remains an open question. The results on the control of the hexose transporter in resting yeast cells (chapter 5) cannot be easily extended to conditions with growing yeast cells, as the expression of carriers depends on the glucose concentration under these conditions.

Genetic manipulation of the number of carriers will show pleiotropic effects due to the interference of glucose signaling, and will yield therefore global (hierarchical) control coefficients. If classical control coefficients are to be obtained (e.g. to assess the regulatory potential of the hexose carrier for the flux distribution between respiration and alcoholic fermentation), fast methods are required. Maltose inhibition of the carrier may be used for this purpose (see chapter 5).

It is feasible, for example, that glucose transport does not control the glycolytic flux to any extent, but that it affects the concentration of intracellular glucose. This might be a signal to repress mitochondrial function. As a result, most of the glucose will be fermented, which requires the glycolytic flux to increase so as to sustain the same growth rate at the lower efficiency of ATP production per glucose consumed. Even though the glucose transporter did not have control on the glycolytic flux in the classical sense, it would have significant control over the glycolytic flux within the context of hierarchical control analysis.

I have suggested that hexose transport does not control the growth rate to a significant extent when yeast is cultivated at high glucose concentrations. Experimental evidence supporting this suggestion has been discussed. Control of the carrier on the glycolytic flux is still open for discussion. It should be noted, however, that high control by that carrier on the glycolytic rate should necessarily reduce the flux control of PFK via the flux summation theorem. This would reduce the impact of effectors acting on PFK.

This has an interesting corollary for the kinetic properties of that transporter. For PFK to have any regulatory strength, the transporter should be sensitive to subsequent steps in metabolism. If the carrier were insensitive to subsequent metabolic steps, it would completely control the flux and hence, the regulatory strength of PFK (and of any other glycolytic enzyme) would reduce to zero. If regulation of the carrier is mediated by intracellular glucose, the elasticity of the carrier for this metabolite should be negative for the hexose transporter to have a positive control on the glycolytic flux (cf. Eq. 9.2). The more negative this elasticity, the lower the control exerted by the carrier (the overall elasticity of the rest of metabolism for intracellular glucose remaining the same).

For low-affinity transport, however, the transport activity measured over the first 5 s after glucose addition was lower than the rate of glucose consumption 5 min later (chapter 4). Low-
affinity transport therefore required to be activated somehow. Similar discrepancies between the fructose uptake rate and the fructose consumption rate have been observed by others [238]. The need for activation appears to be in contradiction with the negative feedback required for positive (or zero) flux control by the low-affinity glucose transporter during growth on high concentrations of glucose. For high-affinity glucose transport no such contradiction appears to exist, as the transport rate always exceeded the flux. Also high-affinity glucose carriers appear to be susceptible to some activation process, however. Under conditions where high-affinity glucose carriers were expressed, mannose was transported with low affinity, and for mannose we found exactly the same problem as for low-affinity glucose transport: the rate of zero trans-influx was lower than the actual mannose consumption rate (chapter 5). Thus the underestimation of the transport kinetics by the zero trans-influx measurements is not related to which HXT is present, but appears to be related to the affinity of the HXT towards the substrate it transports.

A model of the activation of hexose transport, must fulfill the following conditions:

(i) the activation process acts on both high and low affinity hexose carriers.
(ii) the activation process takes more than 5 seconds, perhaps a few minutes, and is only observed when the substrate is transported with a poor affinity. This appears to preclude fast kinetic effects such as binding of hexoses to some activation site.
(iii) The activation process appears to affect $V_{\text{max}}$ more than the $K_{\text{m}}$, which is inferred from calorimetry time-courses. When the substrate was transported with low affinity (at least initially as measured by zero trans-influx), the heat flux decreased almost linearly with time, whereas in the case of high-affinity transport, the heat flux is constant in time, and only drops when the substrate is almost consumed (chapter 3 and 5). This difference is only explained when the affinity of transport remains low during the former experiments.

Activation of carriers has also been observed for the avian erythrocyte glucose transporter GLUT1, where upon addition of metabolic poisons sugar transporters, already present in the cell membrane, were activated [84]. The activation required ATP hydrolysis and may be associated with protein kinase activity. Human GLUT1 forms tetramers or dimers, with similar affinities for glucose [140]. Oligomerization to tetramers increased the uptake rate of 3-O-methylglucose up to 15-fold [415]. GLUT1 activity also appears to be regulated directly by an ATP-binding site. For yeast hexokinase PII a similar type of regulation appears to exist. Hexokinase PII is a phosphoprotein. It is in an equilibrium between monomers and dimers, the equilibrium of which is affected by glucose-induced dephosphorylation of the monomeric form [286]. This process is likely to be involved in glucose signaling. These findings provide quite a few ingredients that may be relevant for the understanding of the activation of yeast hexose transporters.

One possibility is that also hexose transporters in yeast can form oligomers. The distribution between monomers and oligomers may then be affected by hexose-induced (de)phosphorylation. The oligomer should then be more active than the monomer. The monomer
may be active enough to support the observed flux only when it has a high affinity for the substrate it transports, because the monomeric carrier is then largely saturated with substrate and works at $V_{\text{max}}$. For poor-affinity substrates, the monomer would not be active enough and the (need for the) activation process is observed.

If this model applies, it means that also high-affinity glucose transport should become more active in time. This would contradict the simple carrier model for high-affinity glucose transport proposed in chapter 4. The new model requires that the activated transporter is more sensitive to intracellular glucose, i.e. that the carrier is asymmetric (or becomes asymmetric upon oligomerization). Asymmetry would also make the intracellular hexose concentration a more effective inhibitor for low-affinity transport. If that carrier were symmetrical, astronomic concentrations of intracellular glucose (or mannose) would be required to inhibit low-affinity transport significantly.

It is clear that the properties of the hexose carriers are as yet not completely understood. More elaborate kinetic studies, other than zero trans-influx kinetics, are required to solve the problems raised. First, it should be tested whether the transport kinetics do change upon incubation with substrate. This may be done by pulse chase experiments with labeled glucose, during glucose consumption. With consideration of equilibrium exchange effects in the case of a significant concentration of intracellular glucose, the kinetics of label uptake should allow a fair estimation of the kinetics of the carrier during glucose consumption. In view of the suggested model of activation, this would be particularly interesting for high-affinity glucose uptake.

It would appear that the high affinity carrier meets all the conditions required for a proper flux control distribution and a high uptake rate of glucose. Why does the yeast cell bother to make low-affinity transporters all? One answer is that glucose transporters act as glucose sensors, and it makes sense to have different sensors with different affinities to be able to sense the broad range of extracellular glucose concentrations encountered in yeast's natural habitat. SNF3 and RGT2, however, have been discovered to be high and low glucose concentration sensors, respectively [270]. Their presence sheds doubt on the requirement for transporters with different affinities to glucose. On the other hand, in strains expressing only HXT1, a correlation was found between maltase repression and the number of copies of HXT1 present [292]. Thus, glucose-induced repression of maltase was affected even though SNF3 and RGT2 were present and the extracellular glucose concentration was the same. The signaling function of glucose transport may be propagated via a change in the glycolytic flux, or via the concentration of some signaling molecule. In insulinoma cells of the islets of Langerhans, the blood glucose level sensed by glucokinase appears to be propagated via the glycolytic flux [385]. This is not the case in yeast, as demonstrated by Meijer and coworkers [239]. They showed that glucose repression of invertase depends on the concentration of extracellular glucose and not on

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the rate at which glucose is consumed. Thus, repression of maltase was affected by the activity of the hexose carrier at constant glucose concentration, whereas repression of invertase was affected by the concentration of glucose in the medium at constant growth rate. This strongly suggests that some internal signal is involved, at least for the general catabolite-repression pathway. Intracellular glucose is the most likely candidate (chapter 4).

The presence of transporters with different affinities may be rationalized if indeed intracellular glucose is involved in the triggering of glucose-induced regulatory mechanisms. If glucose were transported with constitutive high affinity carriers only, the intracellular glucose concentration would remain constant even when the extracellular glucose concentration would decrease from 1 M to some 10 mM. Indeed, no difference in intracellular glucose was found when 30 or 13 mM glucose was added to cells with high affinity (chapter 4). With constitutive high-affinity glucose transport any mechanism triggered by intracellular glucose would be blind to changes in the extracellular glucose concentrations higher than 10 mM. A low affinity carrier would be able to pick up such a change. With an extracellular glucose concentration of 250 and 30 mM, the intracellular glucose concentrations were 3 and 0 mM, respectively (chapter 4). Thus, the presence of low affinity transport may be required for proper reflection of the extracellular glucose concentration in the intracellular one.

It would be interesting to do competition experiments between a wild type strain and a strain constitutively expressing high-affinity glucose transport at the same maximal rate as the wild type. If low-affinity carriers are merely required for sensing, it should be expected that the growth rate on glucose is not much affected, but that differences will show up when the extracellular glucose concentration is varied. This may reveal whether the presence of low-affinity carriers can have a selective advantage in a milieu where the availability of glucose is variable.

The presence of both high and low affinity carriers seems to be a rather ubiquitous phenomenon. It has been found for phosphate [235], ammonium [230] and iron transport [11], for transport of amino acids such as proline [216] and probably for many other transport systems [253]. It may be interesting to see if also in these cases the presence of the low affinity carrier allows the cells to sense changes in the extracellular environment more quickly than when only high affinity transport systems were available.

9.5 A quantitative approach to biological systems

In this thesis quantitative aspects of the regulation and control of the glycolytic pathway of yeast have been studied. Being quantitative is difficult, as amply exemplified by the complexity described for hexose transport and highlighted in chapter 8 for other glycolytic enzymes. Being quantitative is essential, however, if we want to understand how the television
set works (chapter 1). For this, we need to know the components, the properties of the components and how these components interact. Only in the context of the interactions, can we truly understand the function of the properties of the individual components. Quite a few examples have been provided in this thesis. Oscillations can only be understood in terms of interactions between enzymes, or even between cells. Control of enzymes on systemic behavior depends on the elasticities of all enzymes in the pathway. The difference in regulation of hexokinase in yeast and trypanosomes can be understood when the consequences of the stoichiometric designs of their glycolytic pathways are understood. The kinetic properties of phosphofructokinase may be explained by its role in regulation of carbohydrate metabolism.

Intuition is an important tool for scientists, but it can also lead them astray. Whereas we tend to think in linear relationships, unexpected behavior can arise from the nonlinear relationships that typify biological systems far from equilibrium. Mathematical modeling is a tool that can sharpen or contradict the intuition, generate ideas and point to essential assumptions that should be tested experimentally. The iterative process between modeling and experimental validation is therefore a powerful instrument to refine and deepen our (quantitative) understanding of living organisms. Only then we may hope to understand those fascinating television sets around and within us.