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The Use of *in vivo*-like Enzyme Kinetics in a Computational Model of Yeast Glycolysis

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Abstract

Usually enzyme kinetic parameters are measured under assay conditions that are optimized for a high activity of the enzyme of interest. The aim of this study was to test if the predictive value of a kinetic computer model of yeast glycolysis would be improved by using kinetic parameters measured in a standardized *in vivo*-like assay medium [1]. The V_{max} and some kinetic parameters of the glycolytic and fermentative enzymes were measured in *Saccharomyces cerevisiae* grown in an aerobic, glucose-limited culture. The assays were done both under '*in-vivo*-like' and optimized conditions. The new data were implemented in an adapted version of the glycolysis model of Teusink *et al.* [2]. The '*in-vivo*-like' enzyme kinetic parameters improved the

model substantially as compared to the parameters from optimized assays. In the latter case the model exhibited 'turbo' behaviour, characterized by a dramatic accumulation of hexose phosphates. The *in-vivo*-like kinetic parameters improved the balance between the lower and upper branch of glycolysis and resulted in a better correspondence between model and experiment for both the concentrations of the glycolytic intermediates and the fluxes.

Introduction

Realistic, quantitative computer models of biochemical networks require that the input data are measured under standardized assay conditions. Moreover, the assay conditions need to be representative of the *in vivo* conditions. However, enzyme-kinetic parameters are most often measured *in vitro* and under optimal conditions for each enzyme. In practice this leads to different assay conditions for each enzyme, *e.g.* in different buffers and at different pH and ionic strength [3 – 5]. In a joint effort of the Dutch Vertical Genomics consortium, the European Yeast Systems Biology Network (YSBN) and the STRENDA (Standards for Reporting Enzymology Data) Commission, we have recently developed an assay medium for measuring enzyme activities that closely resembles the cytosolic environment of the yeast *S. cerevisiae* [1]. Most of the V_{max} values measured in this *in vivo*-like assay medium were lower than those measured under optimal conditions for each enzyme, as one should have expected. The only exceptions were aldolase and pyruvate decarboxylase, which had a higher activity under the *in vivo*-like conditions. The V_{max} values of all enzymes were higher than the flux through them under conditions that favour a high glycolytic flux [1]. This is a prerequisite, since the flux can never exceed the V_{max}. Instead, by sub-saturating substrate concentrations or product inhibition, it can easily drop below the V_{max}. Thus, the new data seem realistic and a good starting point for modelling.

Over the last 30 years many kinetic computer models of yeast glycolysis have been constructed. The first models focused mainly on the mechanisms underlying sustained oscillations in yeast cultures and extracts [6 – 10]. The developments in Metabolic Control Analysis (MCA) inspired the construction of a new generation of models to study the distribution of flux control in glycolysis. The applied aim of these models was to amplify or redirect the flux through glycolysis [11 – 14]. The more recent kinetic models were detailed models based on *in vitro* enzyme kinetics [2, 15, 16] and each of these models was developed with a different aim. The model of Rizzi *et al.* [16] was based on published kinetic mechanisms and affinity constants. The enzyme capacities were fitted on data obtained from dynamic experiments of a glucose pulse added to a steady-state culture. Hynne *et al.* modelled the dynamic characteristics of oscillating yeast cultures to estimate the kinetic parameters [15]. Both approaches aimed at *in vivo* parameter estimation. However, the approach of Hynne *et al.* allowed the estimation of not only the enzyme capacities but also their affinity constants [15].

The objective of the modelling by Teusink *et al.* was to evaluate critically to what extent biochemical knowledge from *in vitro* studies could be used to predict the glycolytic flux and the concentrations of glycolytic intermediates [2]. Their conclusion was that the *in vitro* kinetics could not describe the *in vivo* activity for all of the glycolytic enzymes satisfactorily. A model reduction technique that was extensively applied to the dynamics of yeast glycolysis is the linear-logarithmic (lin-log) kinetics approach [17], which is closely related to mosaic non-equilibrium thermodynamics (MNET) [18]. The lin-log kinetic framework showed that with simplified kinetics and less parameters still good model predictions were obtained [19 – 21]. This 'minimalist' approach demonstrated the key importance of the feedback and feed forward loops for glycolytic dynamics [22, 23].

The present study builds on the ideas developed by Teusink *et al.* [2], who used computer modelling as a stringent test of biochemistry. Here we have measured the kinetics of the glycolytic and fermentative enzymes in yeast under *in vivo*-like assay conditions as described in Van Eunen *et al.* [1]. The obtained V_{max} values and some new affinity constants were inserted in the glycolysis model of Teusink *et al.* [2] and it was tested to what extent the *in vivo*-like enzyme kinetics improved the modelling results.

Material and Methods

Growth and sampling

The haploid, prototrophic *Saccharomyces cerevisiae* strain CEN.PK113 – 7D (*MAT*a, *MAL2 – 8*^{c}, *SUC2*, obtained from P. Kötter, Frankfurt, Germany) was cultivated in an aerobic glucose-limited chemostat culture at 30 °C as described in [24]. The dilution rate and hence the specific growth rate of the culture was set at 0.35 h^{-1}. When the culture was at steady state, samples were taken to measure the V_{max} values of all the glycolytic and fermentative enzymes. Subsequently the cells were transferred to anaerobic conditions at a high glucose concentration to measure the maximal glycolytic flux and the intracellular metabolite concentrations. The V_{max} values measured under optimized assay conditions, the flux and metabolite data have been reported before [32].

Glucose-transport activity assay

Zero-trans influx of ^{14}C-labeled glucose was measured in a 5-s uptake assay described by Walsh *et al.* [25] with the modifications of Rossell *et al.* [26] at 30 °C. The range of glucose concentrations was between 0.25 and 225 mM. Irreversible Michaelis-Menten equations were fitted to the data by nonlinear regression.

V_{max} *measurements under* in vivo-*like assay conditions*

Cell-free extracts were prepared freshly by the FastPrep® method described by Van Eunen *et al.* [1]. V_{max} assays were carried out with the prepared extracts via NAD(P)H-linked assays, at 30 °C in a Novostar spectrophotometer (BMG Labtech) as described in detail in [1].

The standardized *in vivo*-like assay medium [1] contained 300 mM potassium, 245 mM glutamate, 50 mM phosphate, 20 mM sodium, 2 mM free magnesium, 5 – 10 mM sulphate, and 0.5 mM calcium. For the addition of magnesium, it was taken into account that ATP, ADP, NADP and TPP bind magnesium with a high affinity. The amount of magnesium added equalled the summed concentration of these coenzymes plus 2 mM, such that the free magnesium concentration was 2 mM. Since the sulfate salt of magnesium was used, the sulfate concentration in the final assay medium varied in a range between 2.5 and 10 mM. Concentrations of substrates and coupling enzymes were as follows:

Hexokinase (HXK, EC 2.7.1.1) – 1 mM NADP, 10 mM glucose, 1 mM ATP and 1.8 U/ml glucose-6-phosphate dehydrogenase (G6PDH, EC 1.1.1.49).

Phosphoglucose isomerase (PGI, EC 5.3.1.9, reverse direction) – 0.4 mM NADP, 2 mM Fructose 6-phosphate (F6P) and 1.8 U G6PDH.

Phosphofructokinase (PFK, EC 2.7.1.11) – 0.1 mM fructose 2,6-bisphosphate, 0.15 mM NADH, 0.5 mM ATP, 10 mM F6P, 0.45 U/ml aldolase (ALD, EC 4.1.2.13), 0.6 U/ml Glycerol 3-phosphate dehydrogenase (G3PDH, EC 1.1.1.8) and 1.8 U/ml triosephosphate isomerase (TPI, EC 5.3.1.1).

Aldolase – 0.15 mM NADH, 2 mM fructose 1,6-bisphosphate (F16BP), 0.6 U/ml G3PDH and 1.8 U/ml TPI.

Glyceraldehyde 3-phosphate dehydrogenase (GAPDH, EC 1.2.1.12, reverse direction) – 1 mM ATP, 0.15 mM NADH, 5 mM 3-phosphoglyceric acid (3PG) and 22.5 U/ml 3-phosphoglycerate kinase (PGK, EC 2.7.2.3).

Glyceraldehyde 3-phosphate dehydrogenase (GAPDH, forward direction) – 10 mM ADP, 1 mM NAD, 5.8 mM glyceraldehyde 3-phosphate and 22.5 U/ml PGK.

3-Phosphoglycerate kinase (reverse direction) – 1 mM ATP, 0.15 mM NADH, 5 mM 3PG and 8 U/ml GAPDH.

Phosphoglycerate-mutase (GPM, EC 5.4.2.1) – 10 mM ADP, 0.15 mM NADH, 1.25 mM 2,3-diphospho-D-glyceric acid, 5 mM 3PG, 2 U/ml enolase (ENO, EC 4.2.1.11), 13 U/ml pyruvate kinase (PYK, EC 2.7.1.40) and 11.3 U/ml lactate dehydrogenase (LDH, EC 1.1.1.27).

Enolase – The activity of ENO was measured by the production rate of PEP, which was analyzed with a spectrophotometer using a wavelength of 240 nm. The assay was measured using the *in vivo*-like assay medium with 6 mM of 2-phosphoglyceric acid (2PG).

Pyruvate kinase – 10 mM ADP, 0.15 mM NADH, 1 mM F16BP, 2 mM phosphoenolpyruvate (PEP) and 13.8 U/ml LDH.

Pyruvate decarboxylase (PDC, EC 4.1.1.1) – 0.2 mM TPP, 0.15 mM NADH, 50 mM pyruvate and 88 U/ml alcohol dehydrogenase (ADH, EC 1.1.1.1).

Alcohol dehydrogenase – 1 mM NAD and 100 mM ethanol.

The affinity constants (K_m) of GAPDH for glyceraldehyde 3-phosphate, NAD and NADH were redetermined in the *in vivo*-like assay medium by varying the substrate concentrations.

Model description

The glycolytic model of Teusink *et al.* [2] was the starting point for this study. The aim of the modelling was *(i)* to predict the steady-state flux and metabolite concentrations, under the conditions of the fermentative capacity assay (high glucose, anaerobic) and at the measured V_{max} values, *(ii)* to compare the model outcome to the measured flux and metabolite concentrations and *(iii)* to test how the *in vivo*-like V_{max} values affected the correspondence between model and experiment, as compared to the V_{max} values from the optimized assays. The model as it was used here, is depicted in Figure 1. Starting from the original Teusink model [2] the following modifications were made, based on new insights and in order to tailor the model to the experimental conditions of this study.

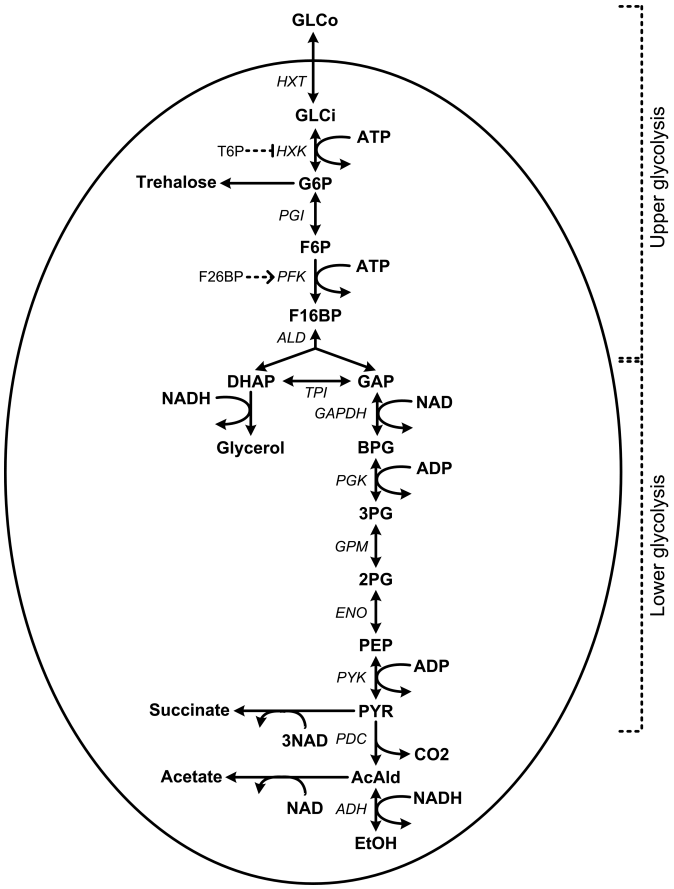

Figure 1. The glycolytic and fermentative pathway as they were modelled in this study. Metabolites are depicted in bold, allosteric regulators in regular, enzymes in italics and branching pathways underlined. GLCo: extracellular glucose, GLCi: intracellular glucose, G6P: glucose 6-phosphate, F6P: fructose 6-phosphate, F16BP: fructose 1,6-bisphosphate, DHAP: dihydroxyacetone phosphate, GAP: glyceraldehyde phosphate, BPG: 1,3-bisphosphoglycerate, 3PG: 3-phosphoglycerate, 2PG: 2-phosphoglycerate, PEP: phosphoenolpyruvate, PYR: pyruvate, ACE: acetaldehyde, EtOH: ethanol, HXT: hexose transport, HXK: hexokinase, PGI: phosphoglucose isomerase, PFK: phosphofructo kinase, ALD: aldolase, TPI: triose-phosphate isomerase, GAPDH: glyceraldehyde 3-phosphate dehydrogenase, PGK: 3-phosphoglycerate kinase, GPM: phosphoglycerate mutase, ENO: enolase, PYK: pyruvate kinase, PDC: pyruvate decarboxylase, ADH: alcohol dehydrogenase.

1. The V_{max} values of all glycolytic and fermentative enzymes and the V_{max} and affinity constant of glucose transport that were measured under the conditions of this study (Table 2) were implemented in the model. For most of the remaining kinetic parameters we have used the values of Teusink *et al.* [2]. The only

exceptions were the kinetic parameters of GAPDH that were re-determined under the *in vivo* like conditions (Table 3). The original GAPDH parameters were used together with the optimized V_{max}, while the newly determined parameters were used together with the *in vivo*-like V_{max}.

2. In the original Teusink model the branching fluxes to trehalose and glycogen were fixed at their measured values. Under the conditions described here, the glycogen flux was negligible and therefore not included. The trehalose flux was fixed at the value measured in this study and so were the fluxes to glycerol and succinate (Table 1). To prevent a redox problem in the model we did not fix the flux to acetate. Instead it was made linearly dependent on the acetaldehyde concentration with a rate constant of 0.5.

3. In the original model the net ATP produced by glycolysis was consumed in a lumped reaction of ATP utilization. This resulted in variable and interdependent ATP, AMP and ADP concentrations. Since information about the kinetics of ATP utilization was lacking and moreover not the focus of this study, we decided to remove the ATP utilization from the model and instead inserted the concentrations of the ATP, ADP and AMP as fixed parameters.

4. The known inhibition of HXK by trehalose-6-phosphate (T6P) was not included in the original model. Yet, it is thought to play an important role in the regulation of glycolysis, particularly to prevent an unbalance between the upper and lower part of the pathway [27]. T6P is an inhibitor of HXK that competes with its substrate glucose. Different K_i values for the different hexokinases of yeast have been reported. Glucokinase was not inhibited by T6P, while the K_i values for hexokinase I and II were 0.2 mM and 0.04 mM, respectively [28]. Here we have used a K_i value of 0.2 mM. The modified kinetic equation of HXK is:

$$v_{hxk} = \frac{V_{\max,hxk} \cdot \left(\frac{Glc_i(t)}{K_{m,hxk,Glci}} \cdot \frac{ATP}{K_{m.hxk,ATP}} - \frac{G6P(t) \cdot ADP}{K_{m,hxk,Glci} \cdot K_{m,hxk,ATP} \cdot K_{eq,hxk}} \right)}{\left(1 + \frac{Glc_i(t)}{K_{m,hxk,Glci}} + \frac{G6P(t)}{K_{m,hxk,G6P}} + \frac{T6P}{K_{i,hxk,T6P}} \right) \cdot \left(1 + \frac{ATP}{K_{m,hxk,ATP}} + \frac{ADP}{K_{m,hxk,ADP}} \right)}$$

5. Finally, the K_m of PDC in the original model [2] was obtained from Boiteux and Hess [29] based on a intracellular phosphate concentration of 25 mM. However, we have measured the PDC activity at a phosphate concentration of 50 mM, which is likely to be the intracellular concentration under the growth conditions studied here [30]. Based on the data of Boiteux and Hess [29] we calculated a K_m value of PDC for pyruvate of 6.36 mM at 50 mM phosphate, and the new value was inserted in the model. For comparison, in the original model the K_m of PDC was 4.3 mM.

   All experimental data were converted to intracellular units (mM min^{-1} for rates and mM for concentrations) by assuming a yeast cytosolic volume of 3.75 µl.mg cell $protein^{-1}$ [31].

Results

Measurements of glycolytic flux, intracellular metabolite concentrations and V_{max}

Yeast cells were grown under aerobic glucose-limited chemostat conditions at a growth rate of 0.35 h^{-1}. When the chemostat cultures were at steady state, cells were harvested to measure *(i)* the maximal glycolytic flux and the intracellular metabolite concentrations in an off-line assay under anaerobic glucose-excess conditions (fermentative capacity) and *(ii)* the V_{max} of the glycolytic and fermentative enzymes. The fluxes and metabolite concentrations, as well as the V_{max} values under optimized assay conditions, were already reported [32] and are taken from the latter study. The V_{max} values under *in vivo*-like conditions and the kinetics of glucose transport are new measurements made for the present study. Tables 1 to 4 show the complete dataset.

Table 1. The measured fluxes through to the side branches, *i.e.* to trehalose, to glycerol and to succinate. The fluxes to these side branches are used as fixed parameters in the model. The data were taken from [32].

	Flux ($mM.min^{-1}$)
Trehalose	1.0 ± 0.3
Glycerol	21.3 ± 0.7
Succinate	0.9 ± 0.0

Table 2. Kinetic parameters measured under optimal and *in vivo*-like assay conditions. Parameters measured under the optimal assay conditions were taken from [32]. The glucose-transport kinetic parameters were measured in intact cells. Since the cells were incubated under the same conditions as used for flux measurements (see Materials and Methods), the results are listed under '*in vivo*-like'. However, the same data for glucose transport were used in both model versions.

Parameter	**Optimal assay conditions**	***In vivo*-like assay conditions**	
$V_{max,glt}$		201.3	$mM.min^{-1}$
$K_{m,glt,GLC}$		0.9	mM
$V_{max,hxk}$	551.9	257.5	$mM.min^{-1}$
$V_{max,pgi}$	1141.3	903.4	$mM.min^{-1}$
$V_{max,pfk}$	98.4	178.7	$mM.min^{-1}$
$V_{max,ald}$	251.0	200.0	$mM.min^{-1}$
$V^{+}_{max,gapdh}$	197.3[a]	156[a]/1496[b]	$mM.min^{-1}$
$V^{-}_{max,gapdh}$	1101.0	866.7	$mM.min^{-1}$
$V^{-}_{max,pgk}$	1662.0	2415.5	$mM.min^{-1}$
$V_{max,gpm}$	1502.7	870.7	$mM.min^{-1}$
$V_{max,eno}$	285.3	485.2	$mM.min^{-1}$
$V_{max,pyk}$	965.4	677.3	$mM.min^{-1}$
$V_{max,pdc}$	218.8	334.7	$mM.min^{-1}$
$V^{-}_{max,adh}$	437.6	856.0	$mM.min^{-1}$

[a] Forward V_{max} values of GAPDH were not measured but calculated with the Haldane relationship using the kinetic parameters from [2].
[b] Forward V_{max} value was measured.

Table 2 shows the V_{max} of the glycolytic and fermentative enzymes measured under both the optimal and the *in vivo*-like assay conditions. In the case of GAPDH, the *in vivo*-like V_{max} was measured in both directions and also the affinity constants were redetermined under *in vivo*-like condition. Since 1,3-bisphoshoglycerate (BPG) is not stable, we could not measure the affinity constant (K_m) for this product. However, we could calculate the K_m for BPG using the Haldane relationship, the measured V_{max}, the measured K_m values for the other substrates and products, and the K_{eq} (Table 3).

Table 3. Kinetic parameters of GAPDH according to Teusink *et al.* [2] and newly measured under the *in vivo*-like assay conditions.

Parameter	According to Teusink *et al.* [2]	Redetermined under *in vivo*-like assay conditions	
$V^{+}_{max,gapdh}$	197.3[a]	1496[b]	$mM.min^{-1}$
$V^{-}_{max,gapdh}$	1101.0	866.7	$mM.min^{-1}$
$K_{m,GAP}$	0.21	0.39	mM
$K_{m,NAD}$	0.09	2.85	mM
$K_{m,NADH}$	0.0098	0.007	mM
$K_{m,BPG}$	0.06	0.51	mM
$K_{eq,gapdh}$	0.0056	0.0056	Taken from [2]

[a] Forward V_{max} values of GAPDH were not measured but calculated with the Haldane relationship using the kinetic parameters from [2].
[b] Forward V_{max} value was measured.

Table 4. Measured intracellular concentrations of the adenine nucleotides and the allosteric regulators of glycolysis that were included in the model. The data were taken from [32].

Metabolite	Intracellular concentration (mM)
ATP	4.29 ± 0.13
ADP	1.29 ± 0.05
AMP	0.44 ± 0.06
T6P	3.52 ± 0.36
F26P	0.004 ± 0.003

The samples for intracellular metabolite measurements had been taken from the incubations for the off-line flux measurements at 15 minutes after the addition of glucose. At that time the measured fluxes had become constant and glycolysis was assumed to be at steady state. The concentrations of the allosteric regulators and those of ATP, ADP and AMP (Table 4) were inserted as fixed parameters in the kinetic model, while the concentrations of the glycolytic intermediates were used as a validation of the model (Table 5).

Table 5. Model results compared to the experimental data. The experimental data were taken from [32].

	Experiment	Model	
		Optimal	*In vivo* New GAPDH parameters
Flux			
HXT-HXK	120 ± 6	84	90
PGI-ALD	99 ± 6	82	88
GAPDH-ADH	177 ± 11	143	155
Metabolites			
G6P	5.4 ± 0.2	**1436.9**	1.9
F6P	1.0 ± 0.0	**378.6**	0.4
F16bP	27 ± 2	**305.6**	27
P3G+P2G	1.2 ± 0.1	0.9	1.2
PEP	0.11 ± 0.01	0.13	0.27
PYR	5.3 ± 0.0	8.7	5.8

Modelling of glycolysis

In order to test the impact of the *in vivo*-like V_{max} values, the V_{max} data measured under both assay conditions were implemented respectively in the adapted kinetic model of yeast glycolysis. Other modifications to the original Teusink model are listed in Materials and Methods.

Figure 2A, C, E and G and the second column of Table 5 show the simulation results of the glycolysis model with the V_{max} data measured under the assay conditions optimized for each enzyme. The model yielded a steady state, however, only at extremely high concentrations for the hexose phosphates in the upper part of glycolysis (Table 5). The concentrations of the metabolites in the lower part of glycolysis came much closer to the experimental data (Table 5).

When the *in vivo*-like V_{max} data were inserted into the model, but not the newly measured *in vivo*-like GAPDH parameters, no steady state was found (data not shown). Fructose 1,6-bisphosphate accumulated, indicating that the lower part of glycolysis failed to keep up with the flux through the upper part of glycolysis. We suspected that this might be due to the GAPDH kinetics. GAPDH is the first enzyme of the lower part of glycolysis and furthermore the kinetics of GAPDH already gave problems in the original model of Teusink *et al.* [2]. Therefore the GAPDH parameters that were re-determined under *in vivo*-like conditions (Table 3) were inserted in the model.

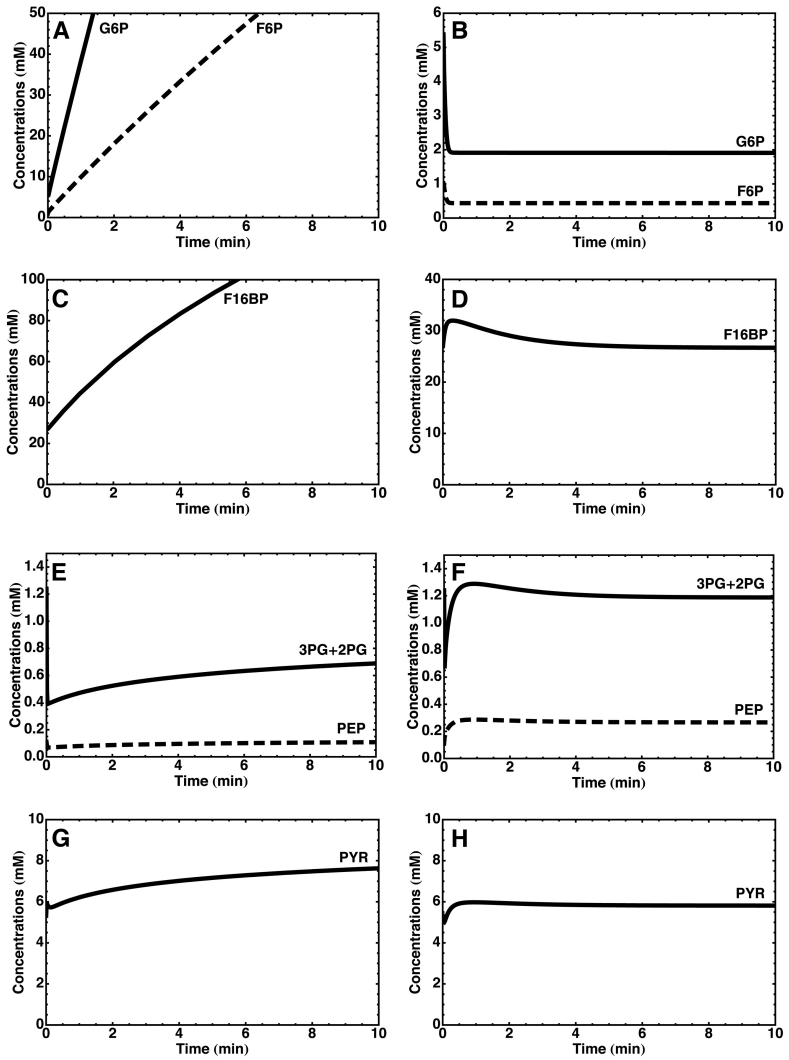

Figure 2. The comparison of the model results of yeast glycolysis obtained with the enzyme-kinetic data measured either in an assay medium optimized for each enzyme (panel A, C, E, and G) or in an assay medium resembling the in vivo situation (panel B, D, F, and H). The concentrations at time zero equal the measured intracellular concentrations. G6P: glucose-6-phosphate, F6P: fructose-6-phosphate, F16BP: fructose-1,6-bisphosphate, 3PG: 3-phosphoglycerate, 2PG: 2-phosphoglycerate, PEP: phosphoenolpyruvate, PYR: pyruvate.

Figure 2B, D, F and H and the last column of Table 5 show the simulation results of the glycolysis model with the *in vivo* V_{max} data and including the new parameters for GAPDH. The steady-state data calculated by the model showed much more similarity with the

experimental data. The fluxes deviated by some 20% between model and experiment (Table 5), yet the best correspondence was reached with the *in vivo*-like V_{max} values. As compared to the model version with optimized kinetic parameters, the most substantial improvement was made in the concentrations of the hexose phosphates in the upper part of glycolysis. They came back to the experimentally observed range and the balance between upper and lower glycolysis was restored.

Conclusion

This paper showed the importance of a standardized enzyme-assay medium which represents the *in vivo* conditions, for realistic quantitative models. In a joint effort of the Dutch Vertical Genomics consortium, the European Yeast Systems Biology Network (YSBN) and the STRENDA (Standards for Reporting Enzymology Data) Commission, such an assay medium was developed for the determination of enzyme-kinetic parameters in the yeast *S. cerevisiae* [1].

The implementation of the *in vivo*-like V_{max} values and the re-determined kinetic parameters of GAPDH has led to an improvement of the glycolytic model. Both fluxes and intracellular concentrations of the glycolytic intermediates showed similarity with the experimental data. The redetermination of the affinity constants of GAPDH was essential, demonstrating that not only V_{max} values but also other kinetic parameters need to be evaluated under the *in vivo*-like assay conditions in future research.

We also tested the impact of the *in vivo*-like parameters when the yeast was grown and maintained under other conditions. Then the correspondence between model and experiment was not quite as good as under the conditions of the present paper (not shown). In ongoing studies it is tested whether redetermination of a larger set of kinetic parameters under the *in vivo*-like conditions will improve the model.

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