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Measuring enzyme activities under standardized *in vivo*-like conditions for systems biology

Karen van Eunen[1,2], Jildau Bouwman[1,2], Pascale Daran-Lapujade[2,3], Jarne Postmus[4], André B. Canelas[2,3], Femke I. C. Mensonides[1,2], Rick Orij[4], Isil Tuzun[5], Joost van den Brink[2,3], Gertien J. Smits[4], Walter M. van Gulik[2,3], Stanley Brul[4], Joseph J. Heijnen[2,3], Johannes H. de Winde[2,3], M. J. Teixeira de Mattos[5], Carsten Kettner[6], Jens Nielsen[7], Hans V. Westerhoff[1,2,8] and Barbara M. Bakker[1,2,9]

1 Department of Molecular Cell Physiology, Vrije Universiteit Amsterdam, The Netherlands
2 Kluyver Centre for Genomics of Industrial Fermentation, Delft, The Netherlands
3 Department of Biotechnology, Delft University of Technology, The Netherlands
4 Department of Molecular Biology and Microbial Food Safety, Swammerdam Institute for Life Sciences, University of Amsterdam, The Netherlands
5 Department of Molecular Micriobial Physiology, Swammerdam Institute for Life Sciences, University of Amsterdam, The Netherlands
6 Beilstein-Institut zur Förderung der Chemischen Wissenschaften, Frankfurt/Main, Germany
7 Department of Chemical and Biological Engineering, Chalmers University of Technology, Gothenburg, Sweden
8 Manchester Centre for Integrative Systems Biology, Manchester Interdisciplinary BioCentre, The University of Manchester, UK
9 Department of Paediatrics, Centre for Liver, Digestive and Metabolic Diseases, University Medical Centre Groningen, University of Groningen, The Netherlands

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Correspondence
B. M. Bakker, Department of Paediatrics, Centre for Liver, Digestive and Metabolic Diseases, University Medical Centre Groningen, University of Groningen, Hanzeplein 1, NL-9713 GZ Groningen, The Netherlands
Fax: +31 50 361 1746
Tel: +31 50 361 1542
E-mail: B.M.Bakker@med.umcg.nl

Note
As a team and independently, the authors are actively engaged in ongoing efforts of the international scientific community to define standards for yeast and other organisms and to get them widely adopted. Hence, the authors would specifically welcome responses from readers who would like to be involved in such efforts and/or have specific comments on the proposed standards or the scientific strategy to define them.

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Realistic quantitative models require data from many laboratories. Therefore, standardization of experimental systems and assay conditions is crucial. Moreover, standards should be representative of the *in vivo* conditions. However, most often, enzyme–kinetic parameters are measured under assay conditions that yield the maximum activity of each enzyme. In practice, this means that the kinetic parameters of different enzymes are measured in different buffers, at different pH values, with different ionic strengths, etc. In a joint effort of the Dutch Vertical Genomics Consortium, the European Yeast Systems Biology Network and the Standards for Reporting Enzymology Data Commission, we have developed a single assay medium for determining enzyme–kinetic parameters in yeast. The medium is as close as possible to the *in vivo* situation for the yeast *Saccharomyces cerevisiae*, and at the same time is experimentally feasible. The *in vivo* conditions were estimated for *S. cerevisiae* strain CEN.PK113-7D grown in aerobic glucose-limited chemostat cultures at an extracellular pH of 5.0 and a specific growth rate of 0.1 h^{-1}. The cytosolic pH and concentrations of calcium, sodium, potassium, phosphorus, sulfur and magnesium were determined. On the basis of these data and literature data, we propose a defined *in vivo*-like medium containing 300 mM potassium, 50 mM phosphate, 245 mM glutamate, 20 mM sodium, 2 mM free magnesium and 0.5 mM calcium, at a pH of 6.8. The V_{max} values of the glycolytic and fermentative enzymes of *S. cerevisiae* were measured in the new medium. For some enzymes, the results deviated conspicuously from those of assays done under enzyme-specific, optimal conditions.

Abbreviations
3PGA, 3-phosphoglyceric acid; ADH, alcohol dehydrogenase; ALD, aldolase; ENO, enolase; Fru6*P*, fructose 6-phosphate; G3PDH, glycerol-3-phosphate dehydrogenase; G6PDH, glucose-6-phosphate dehydrogenase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GPM, phosphoglycerate mutase; HXK, hexokinase; LDH, lactate dehydrogenase; PDC, pyruvate decarboxylase; PFK, phosphofructokinase; PGI, phosphoglucose isomerase; PGK, 3-phosphoglycerate kinase; PYK, pyruvate kinase; STRENDA, Standards for Reporting Enzymology Data; TPI, triosephosphate isomerase.

Introduction

One of the major goals of systems biology is to create comprehensive, quantitative and predictive models that enhance our understanding of cellular behaviour. To achieve this goal, the integration of experimental, computational and theoretical approaches is required [1]. For integration into models and exchange of experimental data from different research groups, it is essential to standardize the cellular systems and experimental procedures [2]. This was done recently for yeast systems biology in The Netherlands by the Vertical Genomics Consortium, consisting of six research groups from three different universities [3], and on a European scale by the Yeast Systems Biology Network (publication in preparation).

However, standardization *per se* is not sufficient. It is crucial that the standards lead to data that are representative of the *in vivo* condition. In the case of pathway fluxes, *in vivo* rates can be measured, and it is also possible to measure absolute concentrations of proteins [4] and transcripts [5] in the cell. However, enzyme–kinetic parameters are currently measured mainly *in vitro* and under optimal conditions for the enzyme under study. Thus, different conditions are used for different enzymes with respect to buffers, ionic strength, etc. [6–8]. As a first step, the Standards for Reporting Enzymology Data (STRENDA) Commission has published recommendations for the unambiguous reporting of enzyme–kinetic data, including a precise description of the assay conditions [9,10]. Strict adherence to these standards in public databases will be of great help in evaluating the data for use in computer models of metabolic pathways. Even more important, however, will be the definition of standard assay conditions that resemble the intracellular conditions in which the enzymes function. This is not straightforward, as the intracellular conditions depend on the environment and cell type, and differ between intracellular compartments.

In this article, the Vertical Genomics Consortium, Yeast Systems Biology Network and STRENDA present a standardized *in vivo*-like assay medium for kinetic studies on cytosolic yeast enzymes. The medium is as close as is reasonably achievable to the *in vivo* situation, according to new measurements and literature data. At the same time, the use of the medium is experimentally feasible, and an identical medium can be used for all enzymes found in the yeast cytosol. The strategy used in this study may serve as a blueprint for standardization of enzyme assays for other cell types and conditions.

Results

Estimation of intracellular ion concentrations on the basis of elemental analysis

Saccharomyces cerevisiae strain CEN.PK113-7D was grown in aerobic glucose-limited chemostat cultures at a dilution rate of $0.1\ h^{-1}$. This strain and cultivation condition were chosen on the basis of earlier standardization attempts [11–14]. First, the biomass composition was determined in samples from these cultures. Table 1 shows the measured amounts expressed in grams of element per kilogram of biomass, and the calculated intracellular concentrations (mM) of the measured elements. The calculated concentrations do not represent free ion concentrations, but average total concentrations of chemical elements. Free ion concentrations were estimated as discussed below. We have used the conversion factors given in Experimental procedures to convert the measurements expressed per dry weight into intracellular concentrations of elements.

Potassium

The concentration of potassium calculated from the elemental analysis was approximately 340 mM (Table 1). Taking into account the experimental error, this is consistent with the literature values, which are between 290 and 310 mM [15–17]. We used 300 mM potassium in the assay medium.

Free phosphate

From the elemental analysis, we could only estimate the total concentration of phosphorus, which was

Table 1. Inductively coupled plasma atomic emission spectroscopy elemental analysis of the biomass. Errors represent standard deviation of two independent chemostat cultures.

Element	Ca	K	Mg	Na	P	S
Measured amount (g per kg dry weight)	0.16 ± 0.07	28 ± 2	2.6 ± 0.0	1.3 ± 0.1	20 ± 1	3.0 ± 0.0
Calculated intracellular concentration (mM)	1.9 ± 0.1	342 ± 30	51 ± 1	28 ± 3	304 ± 14	45 ± 0

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~ 300 mM. A substantial part of this is present in bound phosphate groups or in the form of polyphosphates. To estimate the free cytosolic phosphate concentration, we used values from the literature. A broad range was found, from 10 to 75 mM [14,18–21]. As the growth conditions applied by Wu *et al*. [14] were almost identical to our growth conditions, we used their value of 50 mM. However, we note that varying the phosphate concentration between 10 and 75 mM did not affect the reported V_{max} values (Fig. S1), as reported below.

Sodium

Despite the low sodium concentration in the medium (0.2 mM), the intracellular concentration estimated from elemental analysis was nevertheless 28 mM. In the literature, values of ~ 20 mM were found [15,17]. When reported [15], the extracellular sodium concentration was higher than in our experiments (2 mM), but this still implied a 10-fold accumulation of sodium inside the cells. We note that the CEN.PK strain lacks the sodium efflux pumps encoded by *ENA1–5* [22], which are present in other yeast strains and keep the intracellular sodium concentration low [23]. Instead, it contains a single *ENA6* gene, the expression and/or activity of which is too low for the efficient export of sodium [24]. If we assume only passive sodium transport, sodium should indeed accumulate intracellularly, owing to the membrane potential, which is negative inside. We calculated the plasma membrane potential that would be required to achieve the observed 140-fold accumulation, and obtained −128 mV. This seems a realistic value, as membrane potentials between −50 and −300 mV have been found for fungi [25–28].

Free cytosolic magnesium

The total cellular magnesium concentration as estimated from the elemental analysis was 51 mM. In the cell, most of the magnesium is bound to polyphosphates, nucleic acids, ATP, ADP, etc. [29]. The concentration of free magnesium in the cytosol is unclear, but is estimated to be between 0.1 and 1 mM [30]. It is known that, for the proper functioning of some enzymes, binding of magnesium is essential [29]. As ATP, ADP, etc. were added to the enzyme assays, we decided to add an amount of magnesium such that a free magnesium concentration of 2 mM was obtained. The reason for using a higher free magnesium concentration than is estimated in cells is that it is problematic to prepare a lower free magnesium concentration in a reproducible way, as the free concentration depends on other assay components.

Free sulfate

The total concentration of sulfur calculated from the elemental analysis was ~ 45 mM. In the cell, 90% of the sulfur is present in glutathione [31,32], resulting in a free sulfate concentration of 5 mM. In our assays, sulfate was added to a concentration between 2.5 and 10 mM, depending on the amount of magnesium added, as magnesium was added as magnesium sulfate.

Free calcium

From the elemental analysis, a total calcium concentration of ~ 2 mM was calculated. However, most of the calcium is bound or located in the vacuole [33–35]. Values for free cytosolic calcium found in the literature are very low, between 0.05 and 0.5 μM [36,37]. A problem in dealing with such low concentrations is that traces of calcium are present in glassware, which can cause fluctuating calcium concentrations in the assay. Therefore, we decided to add 0.5 mM calcium to all of the assays.

Cytosolic pH

The measured cytosolic pH was 6.8. The pH chosen for our assay medium was therefore 6.8.

The effect of various anion concentrations on V_{max}

Subsequently, we set out to measure the V_{max} values of the glycolytic enzymes at the intracellular ion concentrations determined above. V_{max} values are key paremeters of kinetic models of metabolic processes (see, for examples of kinetic models, [38–43] and the website JWS Online Cellular Systems Modelling [44]; see http://www.jjj.bio.vu.nl or http://jjj.biochem.sun.ac.za). Here we report total V_{max} (i.e. the summed activity of all isoenzymes present in the cell), expressed per milligram of cell protein, as this is typically used in kinetic models.

If we sum up the concentrations of cations and anions on the basis of the elemental analysis, it is clear that the cation concentration is much higher than the anion concentration. It is known that bicarbonate acts as an anion in the cell [45,46]. However, addition of carbonate to the assay medium is not practical, because of its instability. Amino acids and nucleic acids form substantial groups of anions in the cell. We focused on amino acids to supplement the medium in a practical way. Glutamate is the most abundant amino acid in the cell, and its intracellular concentra-

tion is ~ 75 mM [47]. In all our experiments, we added at least 75 mM glutamate to the assay medium. However, this was insufficient to compensate for the shortage of anions in the medium. Therefore, we tested the effects of the various anion concentrations on the V_{max} values. The three anions tested were glutamate at a concentration exceeding 75 mM, phosphate at a concentration exceeding 50 mM, and the noncellular component Pipes. For the complete medium compositions, see Table 2. Cell-free extracts for these experiments were made in the absence of the phosphatase inhibitors sodium pyrophosphate and sodium fluoride (but see below).

Table 2. *In vivo*-like medium composition with various anion concentrations. Numbers in bold represent the various anion concentrations tested. The total amount of added magnesium depended on the amount of ATP, ADP, NADP, etc. added to the assay. The amount of sulfate depended on the amount of magnesium added to the assay, because sulfate was used as a counterion for magnesium and calcium.

Component	Option 1 (mM)	Option 2 (mM)	Option 3 (mM)
Potassium	300	300	300
Sodium	20	20	20
Free magnesium	2	2	2
Sulfate	2.5–10	2.5–10	2.5–10
Calcium	0.5	0.5	0.5
Glutamate	**75**	**245**	**75**
Phosphate	**163**	**50**	**50**
Pipes	–	–	**120**

Figure 1 shows the V_{max} values of the glycolytic and fermentative enzymes measured in the three different *in vivo*-like media (Table 2). For comparison, the V_{max} values were also measured under assay conditions that had been optimized previously for high activity [8]. The latter set of assays was chosen because it has been used extensively to characterize fermentation in the CEN.PK113-7D strain [8,48,49] and it was the starting point for standardization in the Vertical Genomics Consortium [50,51].

The high-phosphate medium concentration had a significantly negative effect on the enzymes phosphoglucose isomerase (PGI; EC 5.3.1.9), aldolase (ALD; EC 4.1.2.13), triosephosphate isomerase (TPI; EC 5.3.1.1), glyceraldehyde-3-phosphate dehydrogenase (GAPDH; EC 1.2.1.12) and 3-phosphoglycerate kinase (PGK; EC 2.7.2.3). Alcohol dehydrogenase (ADH; EC 1.1.1.1) was the only enzyme on which the high-phosphate medium concentration had a significantly positive effect, albeit small. When we compared the high-glutamate medium with the Pipes medium, only enolase (ENO; EC 4.2.1.11) showed significantly higher activity in the Pipes medium. Because such high free phosphate concentrations (163 mM) are nonphysiological, and Pipes is a noncellular component, we concluded that the assay medium with 50 mM phosphate and 245 mM glutamate in addition to the remaining components (Table 2, option 2) was most suitable. Further experiments were performed in this medium. An additional reason for this choice is that the total amino acid concentration in the cell is ~ 150 mM [47,52], which

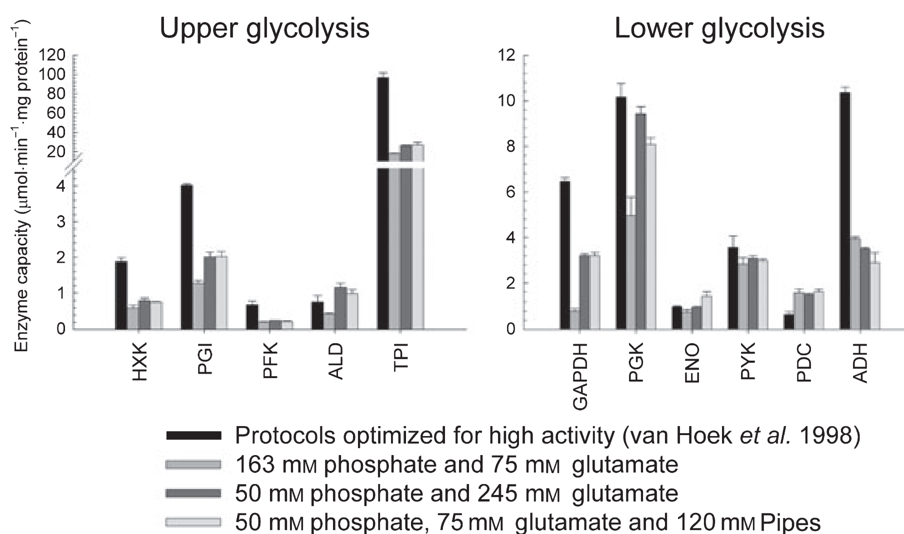

Fig. 1. *In vivo*-like enzyme capacities (V_{max}) measured at various anion concentrations. The V_{max} data obtained with the protocols optimized for high enzyme activity were taken as a reference. Error bars represent standard errors of the mean of at least three independent cell-free extracts from steady-state samples from a single chemostat culture.

compensates substantially, albeit not completely, for the lack of anions. It is therefore realistic and practical to choose the amino acid glutamate as anion in the assay medium. As the precise concentration of free phosphate in the cell was somewhat uncertain (see above), we tested a few concentrations of phosphate. Between 10 and 50 mM, the concentration of phosphate had little or no effect on the measured enzyme activities (Fig. S1).

Table 3 summarizes the V_{max} values measured under optimized conditions (according to Van Hoek *et al.* [8]) and those measured under the definitive *in vivo*-like conditions (Table 2, option 2). Most of the enzymes had a lower V_{max} when measured under the *in vivo*-like conditions than when measured under the optimized conditions. However, for some of the enzymes, e.g. ALD and pyruvate decarboxylase (PDC; EC 4.1.1.1), a higher V_{max} value was obtained in the *in vivo*-like assay medium, suggesting that the 'optimized' conditions are, in reality, not optimal for these enzymes. A thorough analysis of the yeast kinetics of phosphofructokinase (PFK; EC 2.7.1.11) [38] suggested that the concentration of the substrate fructose 6-phosphate (Fru6*P*) (0.25 mM) could have been limiting in our assays. Indeed, a Fru6*P* concentration of 10 mM was sufficient for the V_{max} to be reached. With this substrate concentration, a PFK activity of 0.8 ± 0.1 mmol·min^{-1}·g protein^{-1} was measured (Table 3). Therefore, 10 mM Fru6*P* should be used in future assays.

Table 3. V_{max} values measured under the optimized and the *in vivo*-like conditions in the absence of the phosphatase inhibitors. Errors represent standard errors of the mean of at least three independent cell-free extracts from steady-state samples from a single chemostat culture.

Enzyme	Optimized V_{max} (mmol min^{-1}·g protein^{-1})	*In vivo*-like V_{max} (mmol·min^{-1}·g protein^{-1})
HXK	1.8 ± 0.1	0.80 ± 0.06
PGI reverse	4.0 ± 0.0	2.0 ± 0.1
PFK	0.69 ± 0.10	0.25 ± 0.00 (0.80 ± 0.10[a])
ALD	0.76 ± 0.16	1.2 ± 0.1
TPI	97 ± 5	26 ± 0
GAPDH reverse	6.5 ± 0.2	3.2 ± 0.1
PGK reverse	10 ± 1	9.4 ± 0.3
ENO	0.99 ± 0.04	0.96 ± 0.06
PYK	3.6 ± 0.5	3.1 ± 0.1
PDC	0.65 ± 0.12	1.5 ± 0.1
ADH reverse	10 ± 0	3.5 ± 0.1

[a] V_{max} measured with saturated Fru6*P* concentration for PFK (see text).

The effect of phosphatase inhibitors

To prevent (in)activation of the enzymes by dephosphorylation, phosphatase inhibitors were added before the production of cell-free extracts, and were present throughout the experiment. The phosphatase inhibitors used were sodium fluoride (10 mM) and sodium pyrophosphate (5 mM). Figure 2 shows the V_{max} values measured in the presence and absence of these phosphatase inhibitors. Of all the enzymes, only phosphoglycerate mutase (GPM; EC 5.4.2.1) showed a substantial and significant decrease in activity in the presence of the phosphatase inhibitors. It is known that vanadate, another phosphatase inhibitor, has an inhibitory effect on the activity of GPM from *Escherichia coli* [53].

Can the V_{max} values support the maximal glycolytic flux?

A V_{max} value represents the maximum rate at which an enzyme can work at saturating concentrations of substrates and in the absence of products. In the cell, the flux through the enzyme may be lower than the V_{max}, owing to lower substrate concentrations or product inhibition. The flux through the enzyme can, however, never be higher than the true *in vivo* V_{max}. We therefore tested whether the V_{max} values measured under the *in vivo*-like conditions supported the maximal glycolytic flux that could be reached by cells in which the enzymes were assayed.

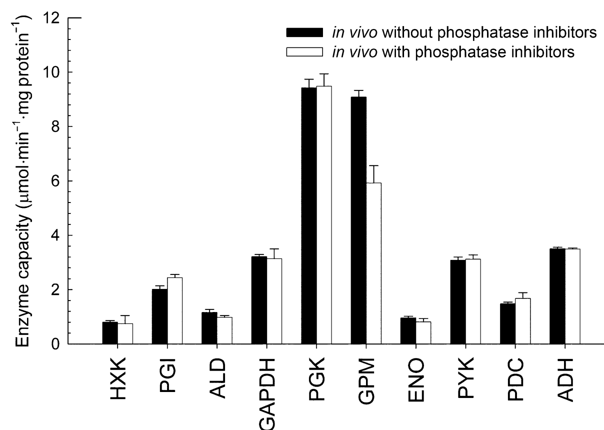

Fig. 2. V_{max} values measured in cell-free extracts made in the presence and absence of the phosphatase inhibitors sodium fluoride (10 mM) and sodium pyrophosphate (5 mM). For these measurements, we have used option 2 as the medium composition (Table 2). Error bars represent standard deviations of at least two independent cell-free extracts from steady-state samples from a single chemostat culture.

Table 4. V_{max} values measured under the *in vivo*-like conditions (in the absence of the phosphatase inhibitors) and the maximal fluxes through the glycolytic and fermentative enzymes. Maximal fluxes were calculated, as described in Experimental procedures, from the offline measured fluxes under anaerobic glucose-excess conditions in steady-state cells from an aerobic glucose-limited chemostat culture at a growth rate of 0.1 h^{-1}. Errors represent standard errors of the mean of at least three independent cell-free extracts from steady-state samples from a single chemostat culture.

Enzyme	*In vivo*-like V_{max} (mmol·min^{-1}·g protein^{-1})	Flux (mmol·min^{-1}·g protein^{-1})
HXK	0.80 ± 0.06	0.35 ± 0.01
PGI	2.8 ± 0.3	0.31 ± 0.00
PFK	0.80 ± 0.10	0.31 ± 0.00
ALD	1.2 ± 0.1	0.31 ± 0.00
TPI	26 ± 0	0.24 ± 0.01
GAPDH	0.59 ± 0.00	0.55 ± 0.01
PGK	111 ± 4	0.55 ± 0.01
GPM	9.1 ± 0.3	0.55 ± 0.01
ENO	0.96 ± 0.06	0.55 ± 0.01
PYK	3.1 ± 0.1	0.55 ± 0.01
PDC	1.5 ± 0.1	0.55 ± 0.01
ADH	56 ± 2	0.55 ± 0.01

The maximal flux was measured under anaerobic glucose-excess conditions in an offline assay using cells from the chemostat cultures. The last column of Table 4 shows the maximal fluxes, calculated for each enzyme individually as described in Experimental procedures. The enzyme capacities were measured in our final assay medium (Table 2, option 2) at a pH of 6.8 in the absence of phosphatase inhibitors. For the enzymes measured in the reverse direction, the V_{max} values were recalculated in the direction of the flux. To obtain these V_{max} values in the catabolic direction, Michaelis–Menten constants and equilibrium constants from the literature were used (ADH [54]; GAPDH [55]; PGI [56]; PGK [57]). The results are shown in Table 4. The *in vivo*-like V_{max} values were sufficient to support the maximal flux.

Discussion

In order to support coordinated efforts to standardize experimental conditions for systems biology, we have formulated an assay medium for kinetic measurements that closely resembles the cytosolic environment of yeast. The assay medium was tested on the glycolytic and fermentative enzymes of *S. cerevisiae*.

The importance of standardization in such a way that it gives rise to realistic *in vivo* parameters cannot be overestimated. The modelling of cellular pathways on the basis of the underlying biochemistry is hampered too often by the fact that kinetic parameters have been measured under nonphysiological conditions. Historically, this is quite understandable, as most enzymology has been aimed at the unravelling of kinetic mechanisms, and for this it is very informative to subject enzymes to extreme conditions. However, data and assay conditions that were chosen for the investigation of catalytic mechanisms cannot be applied directly to models of the *in vivo* behaviour of metabolic pathways. To obtain realistic model predictions, it is crucial to use an *in vivo*-like assay medium that mimics as closely as possible the intracellular environment in which the enzymes function.

The medium that we have developed in this study is representative of the intracellular environment of the yeast CEN.PK113-7D, cultivated under standardized conditions. The question remains of whether such a medium is generally applicable. Within the yeast systems biology community, the CEN.PK113-7D strain is an accepted standard [13], albeit not the only one, and so are the cultivation conditions that we have used here. The same strain and conditions have been used for other standardization efforts, e.g. for transcriptome analysis [12]. Thus, the assay medium will have wide applicability for yeast systems biology. For specific yeast strains or cultivation conditions, or for enzymes localized in other cellular compartments, modifications to the assay medium may be necessary, but even then the medium proposed here is a good starting point. For different organisms or cell types, it will be necessary to develop dedicated assay media. We are aware of and/or involved in such standardization projects for enzyme assays for *E. coli*, lactic acid bacteria and mammalian cells. The procedure described in this article can be followed to develop the most realistic assay medium. In cases where this is not feasible, the yeast assay medium combined with organism-specific literature data still presents a more realistic starting point than the classic assay media for enzyme kinetics.

We are well aware of the fact that the assay medium proposed here has much simpler composition than the cell's interior. We intentionally aimed for simplicity, so that will be feasible to use the assay medium in large-scale (re)determinations of enzyme kinetic parameters. This has necessarily led to compromises. A prominent example is calcium, which we added at a relatively high concentration to avoid fluctuations. An alternative would have been to add an EGTA buffer, but this would have compromised the simplicity of the preparation. Furthermore, some of the ions added to the assay medium vary quite substantially in the cell as a

function of time and conditions. Examples of factors that we know may affect the activity of some enzymes substantially are pH and protons. When such effects are suspected to be important in a specific application, they should be subjected to dedicated studies. The proposed assay medium will then serve as a reference from which variations can be studied systematically. Along similar lines, there are many more metabolites in the cell than in our standardized medium, and each of them may have an effect on the kinetics of a particular enzyme. However, it will be impossible and unnecessary to add them all to the *in vivo*-like medium, because most enzymes will be affected by a limited number of metabolites. Whenever an unknown regulatory effect is suspected, the effect of specific metabolites on the enzyme of interest should be investigated in the context of the *in vivo*-like medium. Finally, *in vivo*, the enzymes are present at much higher concentrations than in typical enzyme assays, in which cell extracts are diluted. The crowded intracellular environment may affect protein–protein interactions and thereby also the activities of the enzymes involved [58]. As an indirect test, we have mimicked the effect of macromolecular crowding on the enzymatic assays by addition of poly(ethylene glycol) or BSA, but we observed no significant effects for the glycolytic enzymes (not shown).

In principle, the new assay medium can be used for all cytosolic enzymes of yeast, and is not limited to glycolytic enzymes. This is because the ions in the medium are, in most cases, not substrates or products of the reactions under study. We must be aware, however, that some of these ions can be converted enzymatically. For instance, for enzymes that convert phosphate or glutamate, it may be necessary to alter the medium composition. Also, we added glutamate as a substitute for amino acids or even anions in general. When glutamate or other amino acids are suspected to be specific regulators, modifications may therefore be necessary. Thus, the standard will serve as an important reference, but critical use is required.

For some enzymes, we observed large differences between their capacities under optimized and *in vivo*-like conditions (Fig. 1). In most cases, the latter conditions yielded lower capacities, as would be expected. Specifically, the activities of a number of enzymes with relatively high V_{max} values (PGI, TPI, ADH) were lower in the *in vivo*-like assay than in the optimal conditions. This makes sense, as protein synthesis is costly for the cell and there is no apparent advantage of disproportional overproduction of a few enzymes. The V_{max} values of all enzymes were higher than the flux through them under conditions that favour a high glycolytic flux. Thus, the new data seem to be realistic and a good starting point for modelling. So far, we have focused on V_{max} values, but other kinetic parameters, such as affinity constants, are also likely to be affected by the composition of the assay medium. We will therefore need to redetermine the affinities of the enzymes for substrates, products and effectors (K_m, K_i, K_a) under the newly formulated assay conditions.

In conclusion, we propose that the assay medium presented here will be a new standard for enzyme activity measurements (i.e. not only glycolytic) in yeast systems biology projects. As discussed above, it will be impossible to stick to a single standard for all future studies, but the strategy followed in this study should serve as a blueprint for a transparent definition of standard assay media.

Experimental procedures

Strain and growth conditions

The haploid, prototrophic *S. 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 in a 2 L laboratory fermenter (Applikon, Schiedam, The Netherlands). The working volume of the culture was kept at 1 L by an effluent pump coupled to a level sensor. Chemostat cultures were fed with defined mineral medium [59] in which glucose (42 mM) was the growth-limiting nutrient, with all other nutrients in excess. Yeast cells were grown under respiratory conditions at a dilution rate of 0.1 h^{-1}. The stirring speed was 800 r.p.m. The extracellular pH was kept at 5.0 ± 0.1 by an Applikon ADI 1010 controller, through automatic addition of 2 M KOH. The fermenter was aerated by flushing with air at a flow rate of 30 $L \cdot h^{-1}$. Chemostat cultures were assumed to be at steady state when, after at least five volume changes, the culture dry weight, specific carbon dioxide production rate and oxygen consumption rate changed by less than 2% upon at least one additional volume change. The number of generations after the start of the chemostat cultivation was kept below 20, because it is known that changes in the cell occur during prolonged chemostat cultivation, to adapt to the limitation conditions [60,61]. In our experiment, samples were taken after 15–18 generations. Cultures were not synchronized with respect to cell cycle, and the samples therefore represent an average of cells in different stages of the cell cycle (as is typical for population samples).

Analytical methods

Culture dry weights were determined as described in [62], with the modification that the filters were dried overnight

in a 60 °C incubator. Cell numbers were counted by a Coulter Counter (Multisizer 3; Beckman Coulter Inc., Fullerton, CA, USA) with a 30 μm aperture.

Elemental analysis

For the elemental analysis of the cytosol, cells were taken from two independent chemostat cultures at steady state. Cells were washed once with demineralized water and freeze-dried. Biomass composition was determined by inductively coupled plasma atomic emission spectroscopy, which was performed by the Energy Research Centre of The Netherlands (ECN, Petten, The Netherlands). The obtained values were converted to intracellular concentrations, on the basis of the following parameters. The biomass dry weight of the cultures was 3.6 $g \cdot L^{-1}$ (measured), which corresponded to 2.5×10^{11} cells L^{-1} (measured). The volume of one cell was taken to be 3×10^{-14} L [63,64].

Cytosolic pH

For measurement of the cytosolic pH, *S. cerevisiae* strain ORY001 was used. This strain has been obtained by transforming CEN.PK113-5D (*MAT*a, *MAL2-8*^{c}, *SUC2 ura3*, from P. Kötter, Frankfurt, Germany) with the plasmid pYES-P_{ACT1}-pHluorin (URA3) [65]. This strain expresses a cytosolic pHluorin, which is a pH-sensitive mutant of the green fluorescent protein [66]. Cells at steady state were directly transferred to CELLSTAR black polystyrene clear-bottomed 96-well microtiter plates (Greiner Bio-One, Alphen a/d Rijn, The Netherlands) to a $D_{600\ nm}$ of 0.5 in defined mineral medium [59] without glucose, and cytosolic pH was measured according to Orij *et al*. (2009).

General procedure for measuring enzyme capacities (V_{max})

For preparation of cell-free extracts, cells were harvested by centrifugation (3850 ***g*** for 5 min at 4 °C), washed twice with 10 mM potassium phosphate buffer (pH 7.5) containing 2 mM EDTA, concentrated 10-fold, and stored at −20 °C. Samples were thawed, washed by centrifugation (3850 ***g*** for 5 min at 4 °C), and resuspended in an equal volume of 100 mM potassium phosphate buffer (pH 7.5) containing 2 mM $MgCl_2$ and 1 mM dithiothreitol. Cell-free extracts were prepared in the presence or absence of the phosphatase inhibitors sodium fluoride (10 mM) and sodium pyrophosphate (5 mM). Cell disruption was achieved by the FastPrep method with acid-washed glass beads (425–600 μm; Sigma Aldrich, St Louis, MO, USA). Eight bursts of 10 s at a speed of 6.0 $m \cdot s^{-1}$ were applied. In between the bursts, samples were cooled on ice for at least 1 min. V_{max} assays were carried out with freshly prepared extracts via NAD(P)H-linked assays, at 30 °C in a Novostar spectrophotometer (BMG Labtech, Offenburg, Germany). The reported V_{max} values represent the total activity of all isoenzymes in the cell at saturating concentrations of the substrates and expressed relative to total cell protein.

Four different dilutions of the extract were used, to check for linearity of the assays. In nearly all cases, two or three dilutions were in the linear range, and these were used for further calculation. Linearity depended strongly on the activity of the enzyme; that is, when the activity was high, the less diluted samples were not linear with the rest of the dilutions. In a few cases, the activity of the enzyme was so low that only the undiluted sample could be measured, i.e. phosphofructokinase and hexokinase (HXK; EC 2.7.1.1). All enzyme activities were expressed as moles of substrate converted per minute per milligram of extracted protein. Protein determination was carried out with the bicinchoninic acid kit (BCA Protein Assay Kit; Pierce, Thermo Fisher Scientific, Rockford, IL, USA) with BSA (2 $mg \cdot mL^{-1}$ stock solution; Pierce) containing 1 mM dithiothreitol as the standard.

V_{max} measurements under optimal conditions

The V_{max} of each enzyme was measured under conditions optimized for maximal activity [8]. Briefly, the conditions used for each enzyme were as follows.

HXK activity was measured in an imidazole/HCl buffer (50 mM, pH 7.6) with 5 mM $MgCl_2$, 1 mM NADP, 10 mM glucose, 1 mM ATP, and 1.8 $U \cdot mL^{-1}$ glucose-6-phosphate dehydrogenase (G6PDH; EC 1.1.1.49).

PGI activity was measured in the reverse direction in the presence of a Tris/HCl buffer (50 mM, pH 8.0) with 5 mM $MgCl_2$, 0.4 mM NADP, 2 mM Fru6*P*, and 1.8 U of G6PDH.

PFK activity was measured in an imidazole/HCl buffer (50 mM, pH 7.0) with 5 mM $MgCl_2$, 0.1 mM fructose 2,6-bisphosphate, 0.15 mM NADH, 0.5 mM ATP, 0.25 mM Fru6*P*, 0.45 $U \cdot mL^{-1}$ aldolase, 0.6 $U \cdot mL^{-1}$ glycerol-3-phosphate dehydrogenase (G3PDH; EC 1.1.1.8), and 1.8 $U \cdot mL^{-1}$ TPI.

ALD activity was measured in a Tris/HCl buffer (50 mM, pH 7.5) with 100 mM KCl, 0.15 mM NADH, 2 mM fructose 1,6-bisphosphate, 0.6 $U \cdot mL^{-1}$ G3PDH, and 1.8 $U \cdot mL^{-1}$ TPI.

TPI activity was measured in a triethanolamine buffer (100 mM, pH 7.6) with 0.15 mM NADH, 5.8 mM glyceraldehyde 3-phosphate, and 8.5 $U \cdot mL^{-1}$ G3PDH.

GAPDH activity was measured in the reverse direction in a triethanolamine buffer (100 mM, pH 7.6) with 1 mM EDTA, 1.5 mM $MgSO_4$, 1 mM ATP, 0.15 mM NADH, 5 mM 3-phosphoglyceric acid (3PGA), and 22.5 $U \cdot mL^{-1}$ PGK.

PGK activity was measured in the reverse direction in a triethanolamine buffer (100 mM, pH 7.6) with 1 mM EDTA, 1.5 mM $MgSO_4$, 10 mM ADP, 0.15 mM NADH, 5 mM 3PGA, and 8 $U \cdot mL^{-1}$ GAPDH.

GPM activity was measured in a triethanolamine buffer (100 mM, pH 7.6) with 1.5 mM $MgSO_4$, 10 mM ADP,

0.15 mM NADH, 1.25 mM 2,3-diphospho-D-glyceric acid, 5 mM 3-PGA, 2 $U{\cdot}mL^{-1}$ ENO, 13 $U{\cdot}mL^{-1}$ pyruvate kinase (PYK; EC 2.7.1.40) and 11.3 $U{\cdot}mL^{-1}$ lactate dehydrogenase (LDH; EC 1.1.1.27).

ENO activity was measured in a triethanolamine buffer (100 mM, pH 8.0) with 1.5 mM $MgSO_4$, 10 mM ADP, 1 mM 2-phosphoglyceric acid, 9 $U{\cdot}mL^{-1}$ PYK, and 13.8 $U{\cdot}mL^{-1}$ LDH.

PYK activity was measured in 100 mM cacodylic acid (pH 6.2) with 100 mM KCl, 25 mM $MgCl_2$, 10 mM ADP, 0.15 mM NADH, 1 mM fructose 1,6-bisphosphate, 2 mM phosphoenolpyruvate, and 13.8 $U{\cdot}mL^{-1}$ LDH.

PDC activity was measured in an imidazole/HCl buffer (40 mM, pH 6.5) with 5 mM $MgCl_2$, 0.2 mM TPP, 0.15 mM NADH, 50 mM pyruvate, and 88 $U{\cdot}mL^{-1}$ ADH.

ADH activity was measured in a glycine buffer (50 mM, pH 9.0) with 1 mM NAD and 100 mM ethanol.

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

On the basis of the data from the elemental analysis (Table 1) and the cytosolic concentrations described in the literature, we designed an assay medium that was as close as possible to the *in vivo* situation, and at the same time experimentally feasible. The choices that had to be made are discussed in Results. The standardized *in vivo*-like assay medium contained 300 mM potassium, 75 mM glutamate, 50 mM phosphate, 20 mM sodium, 2 mM free magnesium, 2.5–10 mM sulfate, and 0.5 mM calcium. As compared with the amount of cations in this medium, there is a shortage of anions. We tested the effects of various concentrations of phosphate, glutamate and Pipes in compensating for this shortage. Table 1 shows the three medium compositions that were tested in order to arrive at the final standard: (a) a glutamate concentration of 75 mM and compensation of the remainder with 163 mM phosphate; (b) a phosphate concentration of 50 mM and compensation of the remainder with 245 mM glutamate; and (c) glutamate and phosphate concentrations kept as they were measured, and compensation of the remainder with 120 mM Pipes. Concentrations of substrates and coupling enzymes were kept the same as described in the protocols of the optimized conditions. However, a concentration of Fru6*P* of 0.25 mM appeared to be far too low to saturate PFK (see Results). Therefore, 10 mM was used when mentioned, and this is also recommended for future studies. For the addition of magnesium, it was taken into account that ATP, ADP, NADP and TPP bind magnesium with high affinity (see Results). The amount of magnesium added equalled the summed concentration of these coenzymes plus 2 mM, such that the free magnesium concentration was 2 mM. Because 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.

With hindsight, we noted that some of our coupling enzyme preparations contained ammonium sulfate. A few tests indicated that the effect will probably be small for the glycolytic enzymes in this study. However, in future studies, this should be avoided by dialysis or by the use of enzyme preparations in glycerol.

The assay medium was stored in small batches at 4° C as three separate components: (a) buffer at pH 6.8 containing 0.9 M potassium, 0.735 M glutamate, and 0.11 M phosphate; (b) buffer at pH 6.8 containing 1.5 M sodium and 1 M phosphate; and (c) 0.01 M calcium sulfate. For each assay, a fresh mix of these three components was prepared. No precipitates were observed in the mix.

Maximal glycolytic flux

To determine the maximal glycolytic flux that could be obtained under conditions that favour glycolysis, the cells were washed and taken up in defined mineral medium [59] lacking glucose. Fluxes were measured under anaerobic conditions with excess of glucose (56 mM, added at time 0) for 30 min in a 6% wet weight cell suspension at 30 °C. The setup used was as described in Van Hoek *et al.* (1998), with the modification that the headspace was flushed with water-saturated N_2 (0.6 $L{\cdot}h^{-1}$) instead of with CO_2. Ethanol, glucose, glycerol, succinate, pyruvate, acetate and trehalose concentrations were measured by HPLC analysis [Aminex-HPX 87H 300 × 7.8 mm ion exchange column (Bio-Rad, Hercules, CA, USA), with 22.5 mM H_2SO_4, kept at 55 °C, as eluent at a flow rate of 0.5 $mL{\cdot}min^{-1}$].

The fluxes through the enzymes of the glycolytic and fermentative pathways were calculated from steady-state rates of glucose consumption, and ethanol and glycerol production. The carbon consumed in these assays matched the carbon produced within the experimental error. The flux through HXK equalled the glucose flux. Fluxes through PGI, PFK and ALD were calculated by dividing the sum of the glycerol and ethanol fluxes by two. The flux through TPI was calculated by subtracting the flux to glycerol from the flux through the previous box (PGI to ALD). The fluxes through the enzymes from GAPDH downstream to ADH were taken to be equal to the measured ethanol flux.

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Supporting information

The following supplementary material is available:
Fig. S1. Enzyme capacities (V_{max}) measured at various phosphate concentrations.

This supplementary material can be found in the online version of this article.

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