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Generating short-term kinetic responses of primary metabolism of *Penicillium chrysogenum* through glucose perturbation in the bioscope mini reactor

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Abstract

A first study of the in vivo kinetic properties of primary metabolism of *Penicillium chrysogenum* is presented. Dynamic metabolite data have been generated by rapidly increasing the extracellular glucose concentration of cells cultivated under well-defined conditions in an aerobic glucose-limited chemostat followed by measurement of the fast dynamic response of the primary metabolite levels (glucose pulse experiment). These experiments were carried out directly in the chemostat as well as in a mini plug flow reactor (BioScope) outside the chemostat. The results of the glucose pulse experiments carried out in the chemostat and the Bioscope were highly similar. During the 90 s time window of the pulse experiment, the glucose consumption rate increased to a value twice as high as in the steady state, a much lower increase than observed for the fermenting yeast *Saccharomyces cerevisiae* under similar conditions. Although the observed metabolite patterns in *P. chrysogenum* were comparable to *S. cerevisiae* large differences in the magnitude of the dynamic behavior were observed between both organisms. During the pulse experiment the level of glycolytic and TCA cycle intermediates, and adenine nucleotides changed between two- and five-fold. Furthermore, a highly similar five-fold increase in the cytocolic NADH/NAD ratio could be calculated from two independent equilibrium assumptions (fructose 1,6 bis-phosphate to the pool of 2 and 3PG and oxaloacetate to fumarate with glutamate transaminase). It was also found that the C4 pool (aspartate, fumarate, and malate) became much more reduced due to this increase in NADH/NAD ratio. Equilibrium conditions were confirmed to exist in the hexose-P pool, the glycolysis between F16bP and 2 + 3PG and in the C4 pool of the TCA cycle (fumarate, malate, oxaloacetate and aspartate).

Keywords: *Penicillium chrysogenum*; In vivo kinetics; Metabolome; Glucose pulse

1. Introduction

Microorganisms are increasingly becoming relevant as production platform for the synthesis of a huge variety of chemicals from renewable carbon sources, due to the ever increasing possibilities of genetic modification. This area of research is called metabolic engineering, where the challenge is to identify gene targets which lead to desirable improvement of the production organism (higher yield and rate of product formation, less by-products, etc.). Use of a mathematical model of microbial metabolism is regarded as a suitable method to identify such targets. Knowledge of the relevant metabolic pathways, the involved enzymes, their kinetic properties, and their genetic regulation are required to create such a mathematical model of metabolism. The pathways and specific enzymes involved can be identified from transcript and biochemical analysis. The kinetic properties of the enzymes have usually been obtained from in vitro experiments using purified enzyme. However, it has become increasingly clear that the in vitro kinetic properties often do not apply to in vivo conditions. Main reason for this is that the in vivo environment of the enzyme strongly deviates from the in vitro conditions applied for the measurement of the kinetic parameters (higher substrate and lower enzyme concentrations, absence of other proteins and metabolites, different pH, etc.).

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One of the prerequisites for the construction of meaningful kinetic models of metabolism is therefore that the kinetic parameters of the relevant enzymes are measured under in vivo conditions.

This has been recognized and kinetic studies to elucidate metabolite interaction and control under in vivo conditions have increasingly being used (Theobald et al., 1993, 1997; Rizzi et al., 1997; Visser et al., 2002; Mashego et al., 2006). In these studies the cells are cultivated under well-defined conditions in a chemostat until a steady state is reached. Subsequently this steady-state culture is disturbed by the addition of a certain perturbing agent (e.g., a substrate, activator or inhibitor) where after the induced transient behavior of metabolism is recorded by measuring the concentrations of intra and extracellular metabolites within a certain time interval. This method has a number of advantages. Firstly, the in vivo approach explores the whole metabolic network in one experiment, contrary to the in vitro approach where each enzyme is studied separately; secondly, dynamic data are rich in information; and thirdly, by studying the response of the metabolome within a short time interval (~300 s) it is allowed to assume that no changes occur in enzyme levels. Under these conditions only the concentrations of metabolites and allosteric effectors influence the catalytic activities of the enzymes. Recently a dedicated device (BioScope) has been developed to carry out short-term pulse response experiments (Visser et al., 2002). With this device perturbation of the steady-state chemostat itself (the conventional approach) is avoided and only a small flow of broth is withdrawn and perturbed outside the chemostat. The BioScope system has several advantages (Visser et al., 2002), e.g., a large number of different perturbation experiments can be carried out using biomass from the same chemostat and thus with identical properties; the system can be applied to study in vivo kinetic properties of cells cultivated in fed batch and large-scale fermentations; only a small amount of perturbing agent is required and the sample volume is in principle unlimited.

Recently (Mashego et al., 2006) an improved BioScope has been developed and used for pulse experiments to study the in vivo kinetics of *Saccharomyces cerevisiae*. Another relevant industrial microorganism is the filamentous fungus *Penicillium chrysogenum*, which is applied for the production of β-lactam antibiotics. In a previous study metabolic flux balancing has been applied to study primary metabolism and its interaction with the penicillin production pathway (van Gulik et al., 2000, 2001). From this study it has been found that penicillin production alters the fluxes through primary metabolism especially with respect to the pentose phosphate pathway and ATP metabolism. Kinetic modeling of the relevant parts of primary metabolism and their interaction with the penicillin biosynthesis pathway should give more insight into how primary and secondary metabolism in *P. chrysogenum* are interconnected.

This work is a first attempt to generate dynamic data for elucidation of the in vivo kinetic properties of primary metabolism of *P. chrysogenum* using stimulus response experiments. Moreover these experiments are carried out in the newly developed Bioscope and the results are compared with results from similar experiments carried out in the chemostat. A comparison is made with the dynamic response of *S. cerevisiae* in a similar glucose pulse experiment.

2. Material and methods

2.1. Strain

A high-yielding strain of *P. chrysogenum* (code name DS12975), was used for all experiments. This strain was kindly donated by DSM Anti-Infectives, Delft, The Netherlands.

2.2. Chemostat cultivation

The chemostat used was a 7-L turbine-stirred bioreactor (Applikon, Schiedam, The Netherlands) with a working volume of 4 L. The chemostat was operated at a dilution rate $D = 0.05\,h^{-1}$. The culture was aerated with 9.33 mol of air per hour (= 0.925 vvm under the applied conditions). Mixing of the reactor content was accomplished with two six-bladed Ruston turbine impellers (diameter 8 cm) operated at a rotation speed of 500 rpm. Foam formation was suppressed by addition of approximately 70 µl/h of anti-foam agent BC86/013 (Basildon Chemicals, Abingdon, UK).

The temperature of the reactor was kept at $25.0 \pm 0.1\,^{\circ}C$ by means of a thermocirculator. The pH of the culture was maintained at 6.5 with 4 N NaOH by an automatic pH-control system (Applikon) using a sterilizable pH probe mounted in the reactor. The dissolved oxygen tension was monitored but not controlled. During the experiments the dissolved oxygen tension never dropped below 50% of air saturation. The concentrations of O_2 and CO_2 in the off gas were analyzed using a combined paramagnetic/infrared gas analyzer (NGA 2000 MLT 1, Fisher-Rosemount GMbH&Co Hasselroth, Germany). Chemostat cultures were directly inoculated with spores grown on rice grains. This was done by gently shaking 10 g of spore containing rice grains in 100 ml of sterile demi water until most of the spores were released. After removal of the rice grains by sieving, the suspended spores were transferred to the chemostat. Chemostat cultivation was preceded by a batch phase. Shortly before the end of the batch phase, e.g., shortly before exhaustion of the carbon source, the feed was started.

2.3. Feed medium

The medium was designed for a biomass concentration of 6 g/L dry weight and contained 16.5 g/L glucose $\cdot H_2O$, 1 g/L KH_2PO_4, 5 g/L $(NH_4)_2SO_4$, 0.5 g/L $MgSO_4 \cdot 7H_2O$, and 10 ml/L of trace element solution. The trace element

solution contained 15 g/L Na_2-EDTA · $2H_2O$, 0.5 g/L $CuSO_4 \cdot 5H_2O$, 2 g/L $ZnSO_4 \cdot 7H_2O$, 2 g/L $MnSO_4 \cdot H_2O$, 4 g/L $FeSO_4 \cdot 7H_2O$ and 0.5 g/L $CaCl_2 \cdot 2H_2O$. For penicillin-G production the side chain precursor phenylacetic acid (PAA) was added at a concentration of 0.94 g/L. The preparation and sterilization of the cultivation medium was carried out as described previously (van Gulik et al., 2000).

2.4. Bioscope system

The BioScope system is based on a mini plug flow reactor (Visser et al., 2002). During operation a small flow of broth is continuously withdrawn from the chemostat and is pumped into the BioScope channel where it is mixed with a continuous flow of perturbing agent, e.g., a glucose solution. In the original version of the Bioscope a silicon tube channel was used, configured as a serpentine system. In a newer design of the device the serpentine channels were milled in a Perspex block (Mashego et al., in press). In this new design the circular channel was divided into two hemi-circular channels by a silicone membrane, i.e., a gas and a liquid channel. The broth from the chemostat is pumped through one of the channels, while a continuous flow of gas is introduced in the opposite channel at the other side of the membrane. O_2 and CO_2 are exchanged via the membrane with the gaseous phase in the opposite channel. This design appeared to be more robust and easy to operate, showed good plug flow characteristics and a large aeration capacity.

The hemispheric gas and liquid channels of the BioScope system used had a diameter of 1.2 mm, and a total length of 6.5 m, corresponding to a volume of 3 ml for each channel. The gas channel was flushed with normal air at a flowrate of 130 ml/min. The broth flow rate used was 1.8 ml/min and the perturbation solution, which contained 5 g/L of glucose, had a flow rate of 0.2 ml/min. This increased the glucose concentration at the entrance of the Bioscope from 20 mg/L, which is the residual glucose level in the chemostat, to 520 mg/L. Broth samples were obtained at residence times of 2, 6.8, 10.5, 15.3, 21.6, 27.7, 37.7, 48.1, 69 and 91.7 s for sampling ports 2–11, respectively. Sample port 1 was located just before the entrance of the glucose solution, thus from this port samples from the steady-state chemostat broth were obtained.

The whole BioScope system, including pumps and glucose solution bottle, was placed in a temperature-controlled cabinet kept at the same temperature as the chemostat (25 °C).

2.5. The broth recycle loop

In order to avoid the entrance of air bubbles from the chemostat into the BioScope channel, a broth recycle sample loop was constructed (see Fig. 1). This recycle loop was driven by a pump (P_1) withdrawing fermentation broth at one side and returning it into the chemostat at the other side (L_2 part in Fig. 1). To minimize the residence time of the broth in the recycle loop a high pumping speed was chosen and the loop was kept as short as possible. Directly behind the entrance of the recycle loop, part of the broth is fed to the BioScope via L_1 while the remainder is pumped back into the chemostat via L_2. Because the flow rate of the broth in the recycle loop is much higher than the flow rate in L_3, that is towards the BioScope, all air bubbles remain trapped in the recycle stream.

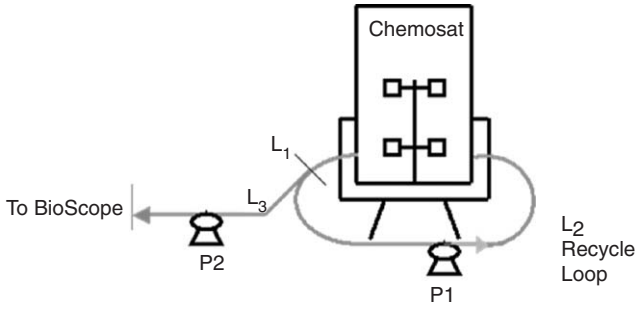

Fig. 1. Schematic drawing of the recycle loop system.

Table 1
Dimensions, flow rates and calculated residence times for the recycle loop system

	Length (cm)	Volume (ml)	Rate (ml/s)	Residence time (s)
L_1	10	0.23	2.36	0.10
L_2	85	7.80	2.36	3.31
L_3	47	0.29	0.03	9.67

A detailed overview of the volumes, flows, and residence times in the different loops is given in Table 1.

2.6. Chemostat pulse experiments

For the pulse response experiment carried out in the chemostat 16 ml of a 125 g/L glucose solution was injected into the reactor within 1 s. This increased the residual glucose concentration to approximately 0.5 g/L. Subsequently, rapid sampling of broth for measurement of intracellular metabolites was carried out at 5, 10, 15, 20, 25, 35, 50, 70 and 90 s after injection of the glucose solution. Rapid sampling and quenching was carried out as described previously (Lange et al., 2001). Sampling times were chosen similar to the sampling times applied for the BioScope experiments. Rapid sampling for determination of extracelluar metabolite concentrations was carried out according to Mashego et al. (2003).

2.7. Bioscope pulse experiments

In order to avoid clogging, water at 25 °C was pumped through the BioScope channel for a period of about 3 min before starting the experiment. Subsequently culture broth and glucose solution (5 g/L) were fed to the BioScope at flow rates of 1.8 and 0.2 ml/min, respectively. The sampling

sequence was started after operating the BioScope for a period of at least 3-residence times (9 min) to let the system stabilize. Sampling was carried out from back to forth, that is from port 11 to 1 and was performed automatically by means of computer-controlled valves. In this way, 1 ml of sample was withdrawn via every sampling port and was instantaneously quenched in a 60% (v/v) methanol/water solution at −35 °C. Further details on the sampling and quenching method have been described previously (Visser et al., 2002).

2.8. Metabolite extraction

Metabolite extraction was carried out with the hot ethanol method (Gonzalez et al., 1997), which was adapted for the extraction of *P. chrysogenum* cells. The adapted methods for cold centrifugation and washing of the cells, metabolite extraction and sample preparation have been described elsewhere (Nasution et al., 2006).

2.9. Analytical procedures

The biomass dry weight concentration was measured by filtration of 10 ml of culture broth on a pre-weighed glass fiber filter (type A/E, Pall Life Sciences, Ann Arbor, MI, USA) and drying to constant weight (24 h at 70 °C).

The concentrations of the glycolytic and TCA cycle intermediates in the cell extracts were analyzed with isotope dilution mass spectrometry (IDMS) as described in Wu et al. (2005). Intracellular nucleotides were analyzed using LC-ESI-MS/MS. The nucleotides were separated by an ion pairing reversed phase HPLC method, using a XTerra MS C18 column (100 mm × 1 mm) equipped with guard column (10 mm × 2.1 mm) (both from Waters, Milford, USA) as described previously (Wu et al., 2005).

Concentrations of PAA and PenG in the culture supernatant were measured with high performance liquid chromatography (HPLC) as described in Christensen et al. (1994). The glucose concentration in the culture supernatant was analyzed spectrophotometrically (Agilent 8453 UV-Visible Spectroscopy System, Waldbronn, Germany) using a Boehringer Mannheim enzymatic bioanalysis kit according to the manufacturer's instructions. The concentrations of organic acids (citrate, pyruvate, succinate, lactate and acetate) in the culture supernatant were determined by HPLC analysis with Aminex® HPC-87H Column (Biorad, Hercules, USA) at 60 °C with 5 mM H_2SO_4 as the mobile phase.

3. Result and discussion

3.1. Steady-state chemostat cultures

For pulse response experiments it is essential to start from reproducible and well-defined cultivation conditions. To achieve this glucose-limited chemostat cultivations were carried out under standardized conditions at a growth rate $\mu = 0.05\,h^{-1}$. From the measured time patterns of the biomass dry weight and penicillin concentrations, and the O_2 and CO_2 concentrations in the offgas during chemostat cultivation of this *P. chrysogenum* strain under the standardized conditions, it was observed that all measured concentrations reached a stable value after six residence times and did not significantly change until the experiment was terminated (results not shown). Table 2 shows the steady-state characteristics of the chemostat in terms of measured concentrations and calculated biomass specific conversion rates. From these rates it was calculated that the carbon and electron balances close for about 90–95%. The most probable reason for the 5–10% gap in the carbon and electron balances, is that not all compounds present in the supernatant have been measured, in particular polymeric byproducts (proteins and polysaccharides) and by-products of penicillin biosynthesis. From previous measurements of the concentrations of these compounds in the culture supernatant under comparable cultivation conditions (van Gulik et al., 2000) it was calculated that the excretion of these compounds indeed represents a gap of

Table 2
Example of measured concentrations and calculated specific conversion rates for a steady-state glucose-limited chemostat of *P. chrysogenum* operated at a dilution rate of $D = 0.05\,h^{-1}$

	Concentration	Specific conversion rate
Biomass	6.21 ± 0.16 g/L	0.051 ± 0.001
Penicillin G	1.81 ± 0.06 mM	0.414 ± 0.02
PAA	2.86 ± 0.09 mM	0.46 ± 0.03
Glucose	0.15 ± 0.06 mM	19.1 ± 0.6
O_2 in off gas	20.46 ± 0.03% (v/v)	48.5 ± 2.3
CO_2 in off gas	0.52 ± 0.08% (v/v)	50.5 ± 2.3

All specific conversion rates are expressed in mmol Cmol/biomass h.

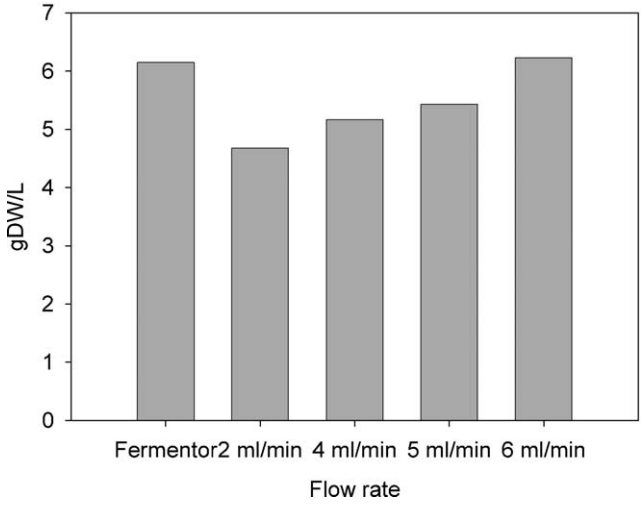

Fig. 2. Measured biomass concentrations at the sample ports of Bioscope at different flow rates using a direct connection between Bioscope and fermentor. The displayed biomass concentrations are averages of biomass samples from all 11 sampling ports.

Table 3
Measured biomass concentrations at the different ports of the BioScope system with recycle loop

Chemostat (g/L)	BioScope sample port (g/L)											
	1	2	3	4	5	6	7	8	9	10	11	Average
6.18±0.09	6.22	6.19	6.47	6.39	6.56	6.38	6.53	5.94	6.49	6.40	6.56	6.41±0.24

5–10% in the carbon and electron balances. This was confirmed by performing a total organic carbon (TOC) analysis of the supernatant of our *P. chrysogenum* chemostat culture (results not shown).

3.2. Chemostat recycle loop system

In first instance a simple direct connection between chemostat and Bioscope was used. However, from measurement of the biomass concentration in broth samples taken from the different ports of the Bioscope large differences were observed with the steady-state biomass concentration in the chemostat (Fig. 2). It can be seen from this figure that at lower flow rates the biomass concentration in the broth from the Bioscope was significantly lower than in the fermentor. Especially at the intended flow rate of 2 ml/min the difference was largest. Apparantly part of the biomass was retained in the tubing connecting the BioScope with the chemostat. In an attempt to solve this problem a recycle loop system was introduced (Fig. 1) which should prevent retention of biomass by fast recirculation of the broth. This appeared to be a significant improvement as can be inferred from the measured biomass concentrations displayed in Table 3. From a comparison of the average biomass concentration measured at the 11 BioScope sampling ports with the biomass concentration measured in the chemostat there appeared to be no significant difference. From the applied tube volumes and flow rates (see Table 1) it was calculated that the residence time of the broth in the tube system between the chemostat and the Bioscope would be approximately 10 s. Using the experimentally determined consumption rates of glucose and oxygen (Table 2) it was calculated that within this time period the residual glucose concentration would decrease from a value of 20 mg/L in the chemostat, to approximately 18.4 mg/L at the entrance of the BioScope. In a similar way it was calculated that the dissolved oxygen level, which is equal to 0.28 mol/m^3 in the chemostat, would decrease to approximately 0.26 mol/m^3 at the entrance of the BioScope. Hence, the calculated decrease in the dissolved oxygen level and residual glucose concentration are relatively small, i.e., 7% and 8%, respectively, and were not expected to have a significant influence on the physiological state of the cells. As an independent check for possible changes in intracellular metabolite concentrations of biomass directly sampled from the chemostat and biomass withdrawn from the first sampling port of the Bioscope (which is before entrance of the glucose solution), were compared (Table 4). It can be seen from this table that most metabolite levels are comparable.

Table 4
Steady-state concentrations of intracellular metabolites (μmol/g DW) in *P. chrysogenum* cells sampled from the chemostat and from the BioScope (from sample port 1)

Metabolite	BioScope	Chemostat
G1P	0.429±0.036	0.608±0.036
G6P	2.229±0.259	3.391±0.036
F6P	0.515±0.052	0.805±0.026
F16bP	0.491±0.064	0.346±0.003
23PG	0.363±0.015	0.340±0.019
PEP	0.202±0.009	0.227±0.006
Pyr	0.542±0.040	0.482±0.014
αKG	1.170±0.110	1.135±0.023
Succ	0.263±0.010	0.252±0.010
Fum	0.683±0.024	0.659±0.047
Mal	3.430±0.110	2.975±0.331
Cit/Isocit	5.179±0.161	4.504±0.033
ATP	6.107±0.025	—
ADP	0.936±0.004	—
AMP	0.405±0.120	—

3.3. Comparison of pulse experiments in the chemostat and the bioscope

The Bioscope pulse experiment was carried out using broth from a chemostat which had been running for 11 residence times. Approximately 24 h later the same chemostat was used to carry out a second pulse experiment, whereby the complete culture was perturbed by injecting the glucose directly in the fermentor. Fig. 3 shows the comparison of the measured patterns of the extracellular glucose concentration vs. time for glucose pulse experiments carried out in the BioScope and directly in the chemostat. From the relatively slow increase of the glucose concentration during the first 15 s after addition of the glucose pulse to the chemostat it can be concluded that only after 15 s complete mixing of the glucose with the broth is achieved. In contrast to this the mixing in the BioScope appears to be instantaneous, as can be inferred from the measured glucose concentration during the first 15 s. It can also be seen from this figure that the glucose concentration measurements for the BioScope perturbation experiment were too scattered to calculate the glucose uptake rate. Therefore, the glucose uptake rate was

calculated from the chemostat perturbation experiment. Assuming a linear decrease of the glucose concentration in time, the average glucose uptake rate was calculated to be approximately 36 mmol/Cmol h This value is about two times higher than the steady-state value (see Table 2).

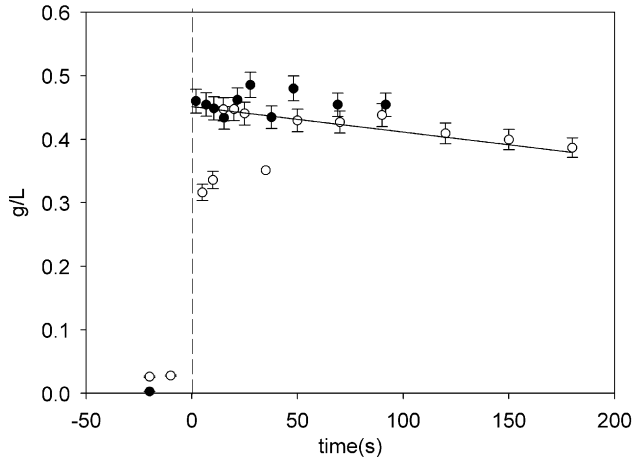

Fig. 3. Extracellular glucose concentration vs. time during glucose pulses in the chemostat (○) and BioScope (●). The linear line used to calculate q_s.

No organic acids (e.g., citric, lactic, succinic, acetic) were excreted during the glucose pulse experiment as these could not be detected by means of HPLC analysis of the supernatant (data not shown).

Fig. 4 shows the measured dynamic time patterns of the glycolytic and TCA cycle intermediates during the pulse experiments. The differences between the Bioscope and fermentor experiments appeared to be small. It can therefore be concluded that the Bioscope is a suitable platform to perform in vivo kinetic experiments with the filamentous fungus *P. chrysogenum*.

3.4. *In vivo kinetic response of primary metabolism of P. chrysogenum to a glucose pulse*

The two-fold increase of the glucose uptake rate after addition of the glucose pulse led to measurable changes in intracellular metabolite levels. It can be seen from Fig. 4

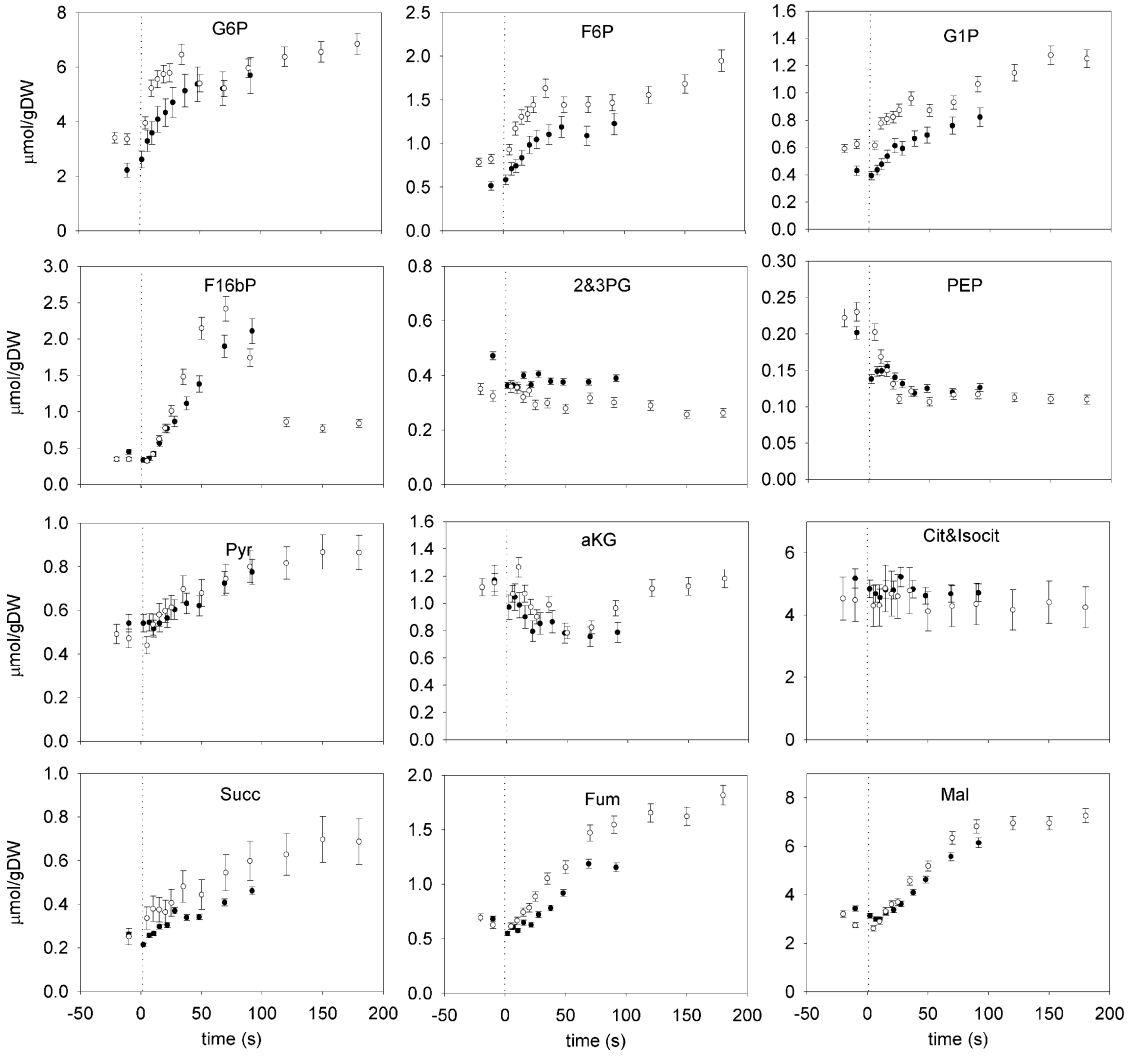

Fig. 4. Response of glycolytic and TCA cycle intermediates to a glucose pulse in chemostat (○) and BioScope (●).

that the levels of the hexose-phosphates G6P, G1P and F6P all increased approximately with a factor 3. Also the level of F16bP increased although the increase was much more pronounced (up to a factor 6). No significant changes were observed for 2 and 3PG, whereas the level of PEP was found to decrease, while the level of pyruvate increased.

Fig. 5 shows the time patterns of the adenine nucleotide levels, which were only measured for the fermentor pulse experiment. It can be seen from this figure that the ATP level decreases linearly to reach a pseudo-steady state at 50 s after addition of the glucose pulse. Both the levels of AMP and ADP show a linear increase until $t = 30$ s. Thereafter a linear decrease is observed for both AMP and ADP to reach a pseudo-steady state, at 70–80 s after addition of the glucose pulse, at levels slightly higher than their steady-state concentrations.

Calculation of the sum of the adenine nucleotides reveals that also in *P. chrysogenum* this sum decreases significantly, as has been observed previously in pulse experiments in *S. cereviseae* (Theobald et al., 1997; Mashego et al., 2006). At 50 s after the glucose pulse the adenine nucleotide sum reached a pseudo-steady state at a value of approximately 85% of the steady-state value. The reason for this rapid net disappearance of adenine nucleotides remains unclear.

It can be calculated from these results that the energy charge during steady-state conditions is high (0.9) and slightly decreases to a value of 0.8 at approximately 40 s after addition of the pulse. However, already after 60 s a slight recovery is observed, although the energy charge did not come back to the steady-state level but reached a plateau at a value of approximately 0.8. A comparable response is observed in similar glucose pulse experiments in *S. cerevisiae* (Mashego et al., 2006). However, in *S. cerevisiae* the energy charge was observed to return to the steady-state level, 60 s after addition of the glucose pulse.

In Fig. 4 the measured time patterns of the TCA cycle intermediates are shown. It can be seen from this figure that the pool of citrate + isocitrate, which is known to be relatively large, does not change significantly during the time window of the glucose pulse experiment. The αKG level was found to decrease to two-third of its steady-state value at 50 s but recovered to reach its steady-state value at 120 s after the glucose pulse. In contrast to this the levels of its C4 products (succinate, malate and fumarate) strongly increased to reach a roughly three-fold higher pseudo-steady-state value at 120 s after the glucose pulse.

3.5. Calculation of mass action ratios

From the measured dynamic metabolite patterns a direct insight in the existence of equilibrium reactions can be obtained. In case of the hexose phosphates an equilibrium was observed to exist for phosphoglucoisomerase and phosphoglucomutase, as can be inferred from the absence of significant changes in the calculated mass action ratios (see Fig. 6). A similar conclusion can be drawn from the calculated mass action ratio for fumarase. The mass action ratio for enolase was observed to decrease slightly after addition of the glucose pulse. The calculation of the mass action ratio for myokinase from the measured concentrations (see Fig. 5) yields values between 0.6 and 0.8, thus slightly higher than the value of 0.4 during steady-state chemostat cultivation. These results indicate that these reactions operated close to equilibrium during the glucose pulse experiment as the calculated mass action ratios for these enzymes appear to remain very close to the known equilibrium constants (Visser et al., 2000 and references therein).

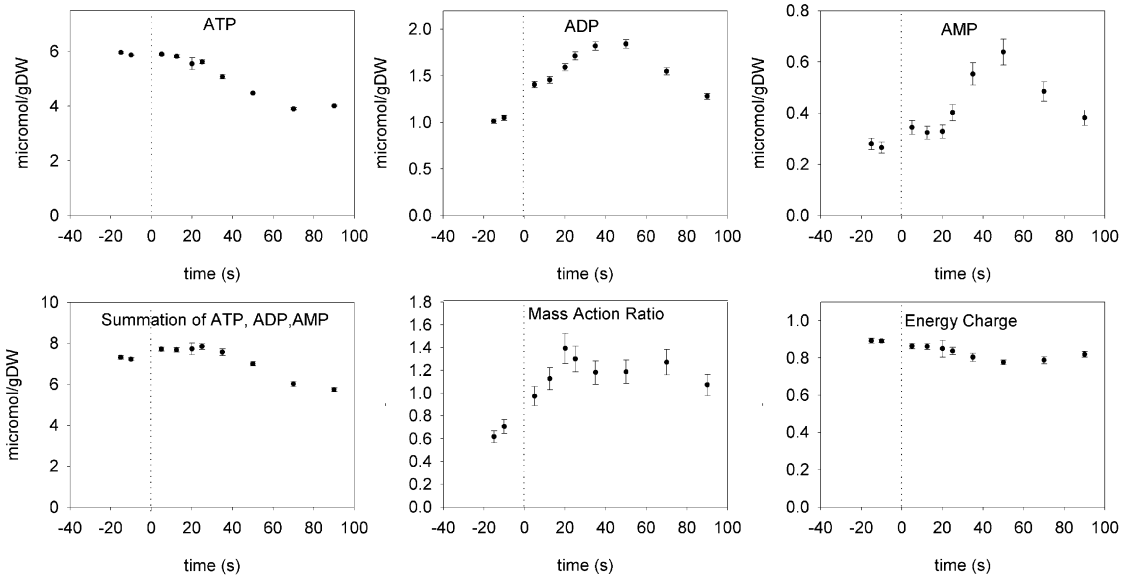

Fig. 5. Upper panels: response of the adenine nucleotides (ATP, ADP, AMP) to a glucose pulse experiment carried out in the chemostat; lower panel: calculated sum of the adenine nucleotides, mass action ratio of adenylate kinase and energy charge vs. time.

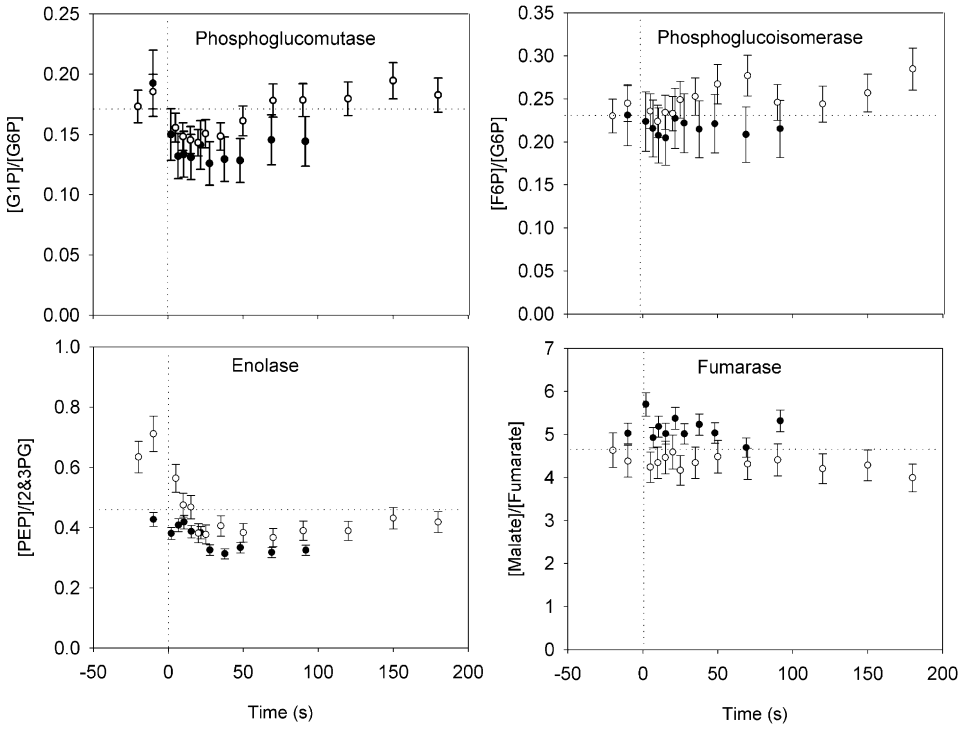

Fig. 6. Calculated mass action ratios of Phosphogluco-isomerase, Phosphogluco-mutase, Fumarase and Enolase in chemostat (○) and BioScope (●) and their equilibrium constant (- - -).

3.6. Estimation of the cytosolic NAD/NADH ratio

As the redox status of the cell directly affects the rates of many reactions it is important to be able to calculate the change of the cytocolic NADH/NAD ratio during pulse response experiments. This can be done by using the assumption that there exists an equilibrium pool between F16bP and 2 and 3PG. The overall reaction of this pool is

$$\frac{1}{2}\,\mathrm{F16bP} + \mathrm{ADP} + \mathrm{P}_i + \mathrm{NAD}^+ \rightleftharpoons (2 \text{ and } 3\mathrm{PG}) + \mathrm{NADH} + \mathrm{H}^+ + \mathrm{ATP}. \quad (1)$$

Assuming an equilibrium constant K, the cytosolic (NADH/NAD) ratio can be written as

$$\frac{\mathrm{NADH}}{\mathrm{NAD}^+} = K\,\frac{\mathrm{P}_i}{\mathrm{H}^+}\left[\frac{\mathrm{ADP}}{\mathrm{ATP}}\right]\left[\frac{(\mathrm{F16bP})^{1/2}}{2\&3\mathrm{PG}}\right]. \quad (2)$$

Using the measured metabolite levels (and assuming constant H^+ and P_i), the fold change of the cytosolic (NADH/NAD) ratio during the pulse can be calculated relative to the steady state. It can be seen from the result shown in Fig. 7 that the cytosolic (NADH/NAD) couple becomes transiently much more reduced as a five-fold increase of the NADH/NAD ratio is calculated. This might be an explanation for the observed transient behavior of the TCA cycle metabolites. It is known that OAA, aspartate, malate and fumarate are connected through

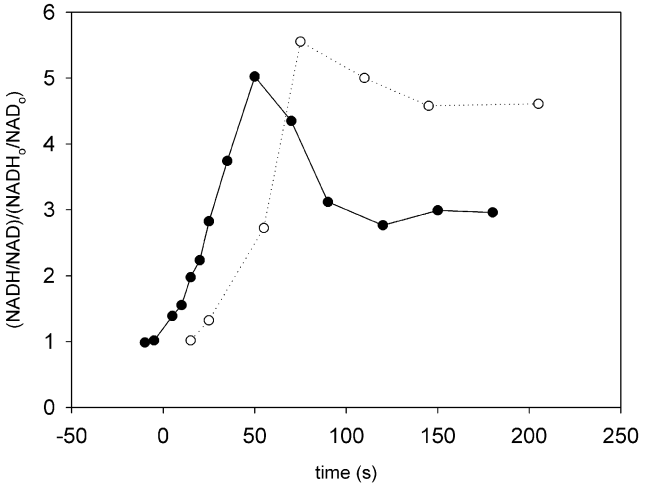

Fig. 7. Fold change in the ratio of NADH and NAD^+ during the glucose pulse experiment relative to the steady-state value calculated from assumed equilibrium between F16bP and 2 and 3PG (●), and calculated from assumed equilibrium between transaminase, malate dehydrogenase and fumarase (○).

reversible enzymes (aspartate transaminase, malate dehydrogenase and fumarase). The overall reaction can then be written as

$$\mathrm{Asp} + \alpha\mathrm{KG} + \mathrm{NADH} + \mathrm{H}^+ \rightarrow \mathrm{Glut} + \mathrm{Fum} + \mathrm{NAD}^+. \quad (3)$$

Assuming equilibrium an alternative expression for the NADH/NAD ratio (with an overall equilibrium constant K') can be derived:

$$\frac{\mathrm{NADH}}{\mathrm{NAD}} = K' \frac{\mathrm{Fum} \cdot \mathrm{Glut}}{\alpha\mathrm{KG} \cdot \mathrm{Asp} \cdot \mathrm{H}^+}. \quad (4)$$

Measurements of the levels of free intracellular amino acids during the pulse experiment (data not shown) indicate that the concentration of glutamate did not significantly change whereas the level of aspartate decreased approximately 20%. Assuming that no changes occur in the intracellular pH, the fold change of the NADH/NAD ratio can also be calculated from these metabolite data using Eq. (4). The result is also plotted in Fig. 7 and appears to be strikingly similar to the fold change calculated from the F16bP to 2 and 3PG equilibrium, in spite of the time delay which is observed when using the latter reaction (Eq. (4)).

The similarity of both calculated patterns indicates that the increase in the C_4-pool (aspartate, malate, fumarate) is caused by the increased reduction status of the NADH/NAD pool in the cytosol.

3.7. Comparison with similar pulse experiment in S. cerevisiae

It is interesting to compare the dynamic response of *P. chrysogenum* to a glucose pulse to the response of *S. cerevisiae* under the same conditions, i.e., growth rate, steady-state biomass concentration and concentration of the glucose pulse (Mashego et al., 2006). Some clear differences have been observed between the responses of the two species to a similar glucose pulse: product secretion does not occur in *P. chrysogenum*, while, due to the Crabtree effect *S. cerevisiae* excretes large amounts of ethanol and acetate (Visser et al., 2004; Mashego et al., 2006). Consequently, the increase of the glucose uptake rate and thus the glycolytic flux observed in *S. cerevisiae* was higher (approximately six-fold) than in *P. chrysogenum* (approximately two-fold). In Fig. 8 the measured dynamics of the glycolytic and TCA-cycle intermediates and the adenine nucleotides during similar glucose pulse experiments in *P. chrysogenum* and *S. cerevisiae* (data from Mashego et al., 2006), both carried out in a chemostat, are compared. It can be seen from this figure that the dynamic patterns of most of the glycolytic intermediates are qualitatively similar for both species. However, in *P. chrysogenum* the changes are much less pronounced, which is most probably caused by the much smaller increase of the glycolytic flux after addition of the glucose pulse. In *S. cerevisiae* the hexose phosphates G6P, G1P and F6P show a very rapid four–six-fold increase after the pulse and return almost to their steady-state values 80 s after addition of the glucose pulse whereas in *P. chrysogenum* the increase is approximately two-fold and the hexose phosphate levels do not return to their steady-state values within the time frame of observation, but slowly increase. In *S. cerevisiae* also the increase of the FbP level and the decrease of 2 and 3PG and PEP are very rapid compared to *P. chrysogenum*, whereas the pyruvate peak observed in *S. cerevisiae* is completely absent. In contrast to the observed differences in the dynamic behavior of the glycolytic intermediates the changes in the levels of the TCA-cycle intermediates, with the exception of αKG, is very similar in both species. The reason for this might be that the changes in the TCA-cycle fluxes are probably much more comparable in both species after the addition of the glucose pulse, as can be inferred from the similar dynamics of the specific oxygen uptake rates of both species during the glucose pulse experiment (results not shown). The cause of the different behavior of αKG remains unclear, but might have been caused by differences in changes of the redox status in both species during the glucose pulse experiments.

Also the dynamic patterns of the adenine nucleotides show similar responses in both species. The ATP level is observed to decrease, although the rate at which this occurs in *P. chrysogenum* is lower than in *S. cerevisiae*. Furthermore the ATP level reaches a pseudo-steady state after 50 s in *P. chrysogenum* where a recovery of the ATP level is observed in *S. cerevisiae*. With respect to ADP and AMP the observed time patterns in both species are very similar, although in *S. cerevisiae* the increase of the AMP level is much less and for ADP almost absent, whereas a pseudo-steady state is reached for both ADP and AMP at a much lower levels. An explanation for the observed differences might be the difference in glycolytic flux. Because the increase of the glycolytic flux induced by the glucose pulse is significantly higher in *S. cerevisiae* the rate of ATP regeneration is also higher. This would lead to the observed faster recovery of the ATP level and lower levels of ADP and AMP in *S. cerevisiae* compared to *P. chrysogenum*.

4. Conclusions

For the first time in vivo kinetic data from rapid pulse response experiments have been obtained for the filamentous fungus *P. chrysogenum*. The measured responses in glucose pulse experiments carried out directly in the chemostat and in the BioScope were very similar. This demonstrates that BioScope can be applied to generate in vivo kinetic data in *P. chrysogenum*. It also demonstrated that the BioScope can be used for filamentous fungi at a considerable biomass concentration. Average changes in metabolite levels as a response to a glucose pulse were two to five-fold in a time frame of 90 s. The measured dynamic patterns of the glycolytic and TCA cycle intermediates and the adenine nucleotides as response to a glucose pulse appeared highly similar to the patterns measured in *S. cerevisiae*, however, the magnitude of the changes was much less extreme. This is probably due to the much lower increase in the glycolytic flux (two-fold) in *P. chrysogenum*

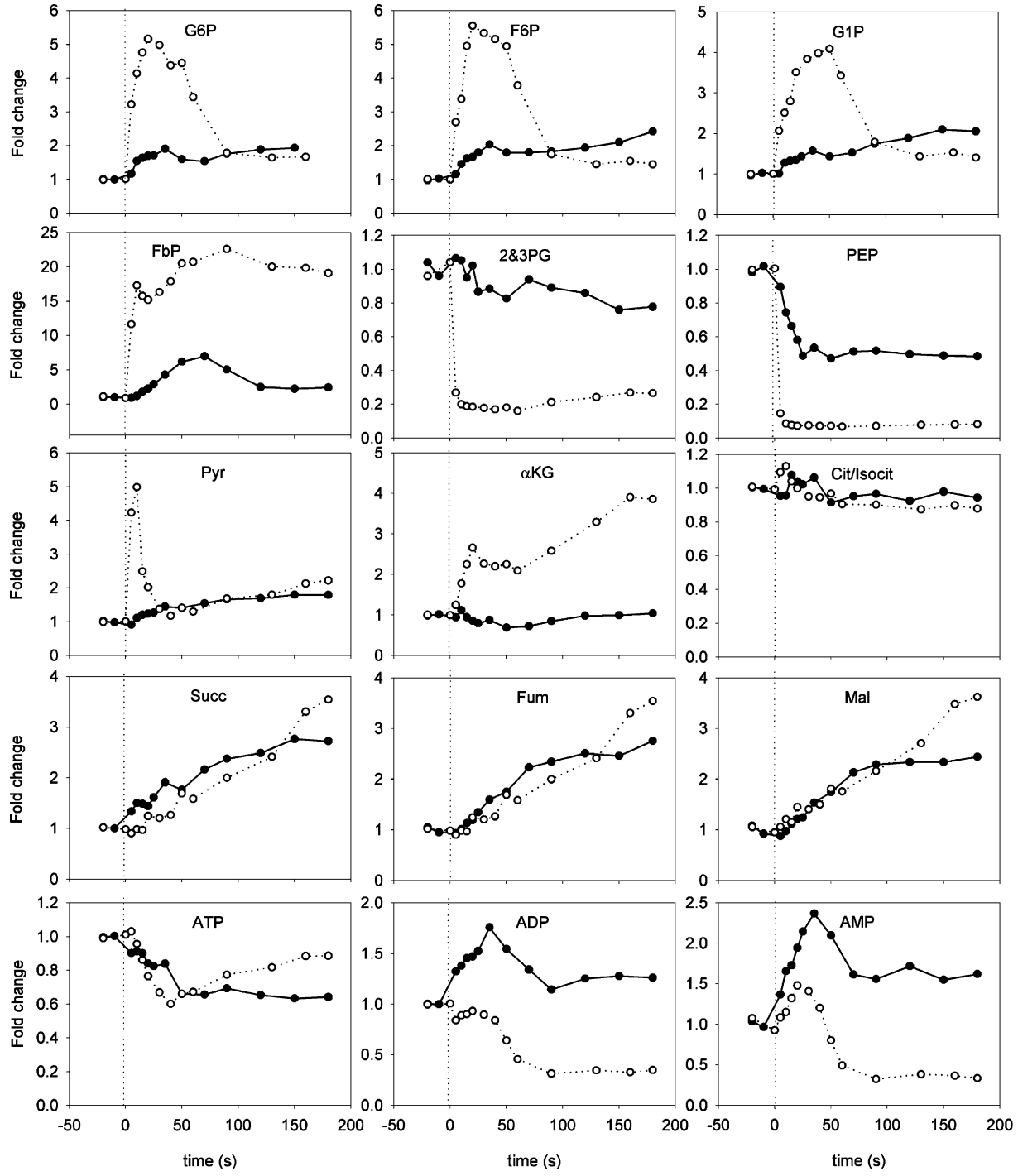

Fig. 8. Comparison of the short-term dynamic response of the glycolytic and TCA cycle intermediates and adenine nucleotides in *P. chrysogenum* (●) and *S. cerevisiae* (○) in similar (with respect to biomass concentration, chemostat dilution rate and glucose concentration) glucose pulse experiments.

than in *S. cerevisiae* (six-fold). In the metabolic network, equilibrium conditions appear to exist in the hexose-P pool, the glycolysis between F16bP and the pool of 2 + 3PG and in the C4 pool (Fumarate, Malate, OAA, Aspartate) of the TCA cycle. The cytosolic NADH/NAD ratio showed a rapid five-fold increase as could be calculated from two independent equilibrium assumptions. These successful pulse response experiments are the first step towards the construction and parameter estimation of an in vivo kinetic model of primary metabolism of *P. chrysogenum*.

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