

In vivo measurement of cytosolic and mitochondrial pH using a pH-sensitive GFP derivative in *Saccharomyces cerevisiae* reveals a relation between intracellular pH and growth

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The specific pH values of cellular compartments affect virtually all biochemical processes, including enzyme activity, protein folding and redox state. Accurate, sensitive and compartment-specific measurements of intracellular pH (pH_i) dynamics in living cells are therefore crucial to the understanding of stress response and adaptation. We used the pH-sensitive GFP derivative 'ratiometric pHluorin' expressed in the cytosol and in the mitochondrial matrix of growing *Saccharomyces cerevisiae* to assess the variation in cytosolic pH (pH_{cyt}) and mitochondrial pH (pH_{mit}) in response to nutrient availability, respiratory chain activity, shifts in environmental pH and stress induced by addition of sorbic acid. The *in vivo* measurement allowed accurate determination of organelle-specific pH, determining a constant pH_{cyt} of 7.2 and a constant pH_{mit} of 7.5 in cells exponentially growing on glucose. We show that pH_{cyt} and pH_{mit} are differentially regulated by carbon source and respiratory chain inhibitors. Upon glucose starvation or sorbic acid stress, pH_i decrease coincided with growth stasis. Additionally, pH_i and growth coincided similarly in recovery after addition of glucose to glucose-starved cultures or after recovery from a sorbic acid pulse. We suggest a relation between pH_i and cellular energy generation, and therefore a relation between pH_i and growth.

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INTRODUCTION

Microbes are able to adapt to a wide range of stressful environments such as deviant temperature, high or low osmotic pressure, oxidative stress and exposure to weak organic acids. The mechanisms by which they adapt to these environments are often poorly understood. To study these adaptive responses we rely on techniques that focus on various levels of cellular regulation, such as transcription profiles, protein levels and metabolic flux analysis. However, global physiological parameters such as intracellular pH (pH_i) affect nearly all processes in a living cell. pH_i directly influences the redox state of the cell by influencing the NAD^+/NADH equilibrium (Veine *et al.*, 1998) and determines pH gradients necessary for transport over membranes (Goffeau & Slayman, 1981; Wohlrab & Flowers, 1982). Additionally, the effect of pH is very prominent in enzyme kinetics, as pH influences ionization states of acidic or basic amino acid side-chains and thereby influences the structure, solubility and activity of most, if not all, enzymes.

The different organelles in the cell all maintain their own specific pH, which is used to define and maintain the

processes associated with each organelle. Yeast vacuoles, for instance, are reported to have an acidic pH (Preston *et al.*, 1989; Brett *et al.*, 2005; Martínez-Muñoz & Kane, 2008). The proton gradient across the vacuolar membrane has been shown to be essential for the transport of various compounds (Nishimura *et al.*, 1998; Ohsumi & Anraku, 1981). The pH of the mitochondrial matrix on the other hand is reported to be alkaline, with a pH of 8.0 (Llopis *et al.*, 1998). This is the result of electron transport chain activity, which pumps protons from the mitochondrial inner matrix, to generate a proton gradient (ΔpH) and an electrochemical gradient ($\Delta\psi$) constituting the proton-motive force used for ATP synthesis. The pH of the peroxisomal lumen is reported to be 8.2; this coincides with the pH optimum for most peroxisomal enzymes, which lies between 8 and 9 (van Roermund *et al.*, 2004). Lastly the secretory pathway has been shown to acidify from 7.2 in the endoplasmic reticulum to 5.2 in the secretory granules. This acidification is necessary for proper protein sorting and modification (Paroutis *et al.*, 2004).

These examples illustrate that organelle-specific pH is a crucial parameter in cell physiology. Therefore, an accurate organelle-specific tool to monitor changes in pH is

Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazonone; pH_{cyt} , cytosolic pH; pH_i , intracellular pH; pH_{mit} , mitochondrial pH.

required to fully understand cellular functioning. Current techniques used to measure the pH are ^{31}P NMR (Gillies *et al.*, 1981; Ogino *et al.*, 1983), probing with pH-sensitive fluorescent dyes (Bracey *et al.*, 1998; Lanz *et al.*, 1999), deploying radioactively labelled membrane-permeable weak acids or bases (Krebs *et al.*, 1983; Siegumfeldt *et al.*, 2000), as well as the equilibrium distribution of benzoic acid (Kresnowati *et al.*, 2007). However, none of these techniques are organelle specific and thus they are bound to result in measurement of an average cellular pH. Additionally, most techniques require extensive manipulation of cells, which itself can affect pH_i (Karagiannis & Young, 2001). A promising method for pH_i measurements is based on *in situ* expression of the pH-sensitive green fluorescent protein ratiometric pHluorin (Miesenböck *et al.*, 1998), which is also functional in yeast species (Karagiannis & Young, 2001; Brett *et al.*, 2005; Martínez-Muñoz & Kane, 2008). In this study we expressed pHluorin in the cytosol and mitochondria of *Saccharomyces cerevisiae* and showed that this allows direct and time-resolved monitoring of the cytosolic and mitochondrial pH (pH_{Cyt} and pH_{mit}) in yeast cells. We studied the effect of nutrient availability on pH_i to verify previously reported data on this subject, and studied how pH_{mit} is affected in fermentative versus respiratory metabolism, and by inhibition of respiratory energy generation. Subsequently, the response of the pH_i to external pH changes was studied, as well as the response to the weak acid sorbic acid. All of these experiments suggested a clear relation between pH_i , the capacity for energy generation and the ability of cells to grow.

METHODS

Strains, media and growth conditions. Strains used in this work are listed in Table 1. Strains were grown at 30 °C in a defined mineral medium as described by Verduyn *et al.* (1992) using either 2% glucose, or different carbon sources specified in the text and legends. The medium was selected because of its low background fluorescence; it is similar to the medium containing yeast nitrogen base (YNB) without folic acid and riboflavin as described by Sheff & Thorn (2004). Pre-cultures were grown in Erlenmeyer flasks on a rotary shaker at 200 r.p.m., in the same medium buffered with 25 mM sodium citrate. Strains were cultivated in 500 ml batch fermenters with a steady airflow (500 ml min^{-1}) and stirring rate (600 r.p.m.). The pH of batch fermenter cultures was controlled by titration with 0.2 M KOH using an Applikon ADI 1030 controller. A Novaspec II spectrophotometer (Pharmacia LKB Biochrom) was used for all optical density measurements of batch cultures. Optical density of

microtitre plate cultures was measured with a SpectraMax Plus384 microtitre plate spectrophotometer (Molecular Devices).

Chemostat cultures were grown in aerobic, carbon-limited 2 l chemostats (Applikon) with a working volume of 1 l at a dilution rate of 0.1 h^{-1} . Pre-cultures were grown overnight in mineral medium with 20 g glucose l^{-1} in shake flasks at 30 °C and 200 r.p.m. The medium used for chemostat cultivation was the same mineral medium as used in batch fermenters, but in this case containing 7.5 g glucose l^{-1} . The stirrer speed was set to 800 r.p.m. while the pH was set to 5.0 and kept constant by automatic titration with 1 M KOH. The temperature of the chemostat was controlled with a heat jacket and a temperature probe. Stirring rate, pH and temperature were kept constant using an Applikon ADI 1010 Biocontroller. The chemostat was aerated by flushing air at 30 l h^{-1} through the culture. Steady states were verified by off gas analysis for oxygen and carbon dioxide and by dry weight measurements.

Dry weight concentration was determined in triplicate by filtering 10.0 ml broth on pre-washed and pre-weighed cellulose acetate membrane filters (pore size 0.45 μm , Schleicher & Schuell MicroSciences). Each filter was washed with 10 ml demineralized water and dried in a 450 W microwave (Whirlpool Promicro 825) for 15 min. Filters were cooled in a desiccator and weighed on an electronic analytical balance (Mettler-Toledo AB104).

Oxygen and carbon dioxide levels in the exhaust gas of the fermenters were monitored online using an oxygen analyser (Servomex Paramagnetic O₂ transducer) and a carbon dioxide analyser (infrared Servomex Xentra 4100 gas purity analyser). To analyse glucose, ethanol, glycerol, succinate, acetate and trehalose, 1.0 ml broth was quickly quenched in 100 μl 35% perchloric acid. Samples were subsequently neutralized with 55 μl 7 M KOH. Culture samples and media samples were analysed by HPLC on a Phenomenex Rezex ROA-Organic Acid H+ column using 7.2 mM H₂SO₄ as mobile phase. Data were included if the carbon balance closed within a 10% error.

Strain construction. Cloning procedures were carried out following standard protocols (Sambrook *et al.*, 1989). All constructs were verified by DNA sequence analysis. To clone the *ACT1* promoter 472 bp of sequence preceding the ATG of the *ACT1* ORF were amplified using primers pACT1-N-Spe (GAAGTCTCAAAAC-CCTTAAAAACATATGC) and pACT1-C-Hin (AGAAAGCTTTGT-TAATTCAGTAAATTTTCGATC) on genomic DNA of the CEN.PK 113-7D yeast strain, introducing *SpeI* and *HindIII* sites. Plasmid pYES2-mtGFP (a kind gift from Dr B. Westermann), containing a mitochondrial targeting sequence (Westermann & Neupert, 2000) was digested with *SpeI* and *HindIII* to introduce the *ACT1* promoter. The pHluorin ORF (Miesenböck *et al.*, 1998) was PCR amplified using primers pGS0012 (AGGATCCATGAGTAAAGGAGAAGAAC-TTTTCA) and pGS0011 (AGAATTCCTATTTGTATAGTTCATCC-ATGCC) on pGEX-2t-pHluorin (a kind gift from Dr G. Miesenböck), introducing *BamHI* and *EcoRI* sites as well as start and stop codons that had been removed in the original construct. This fragment was cloned into pYES2-*PACT1*-mtGFP, generating pYES2-*PACT1*-

Table 1. Yeast strains used in this study

Strain	Genotype	Source
CEN.PK 113-7D (WT)	<i>MATa MAL2-8c SUC2</i>	P. Kötter (Frankfurt, Germany)
CEN.PK 113-5D	<i>MATa MAL2-8c SUC2 ura3</i>	P. Kötter
ORY001	<i>MATa MAL2-8c SUC2 ura3; pYES-PACT1-pHluorin (URA3)</i>	This study
ORY002	<i>MATa MAL2-8c SUC2 ura3; pYES-PACT1-mtpHluorin (URA3)</i>	This study

mtpHluorin. After sequencing this construct we noticed that the L220F mutation described by Miesenböck *et al.* (1998) was not there. However, we were still able to achieve proper ratiometric determinations (see below). The mitochondrial targeting sequence was removed by digestion with *Hind*III and *Bam*HI, sequential blunting with T4 polymerase and religation, yielding pYES2-*P_{ACT1}*-pHluorin. Yeast transformation was carried out according to Schiestl & Gietz (1989).

Microscopy. Cells were grown in Erlenmeyer flasks to an OD₆₀₀ of approximately 1.0 in Verduyn medium containing 2% glycerol and 2% ethanol as carbon source; an OD₆₀₀ of 1.0 corresponded to 3.0×10^7 cells. Then 25 nM MitoTracker Red CMXRos (Invitrogen) was added to the medium and cells were cultured for an additional 30 min. After washing with PBS, cells were transferred to agarose-coated glass slides. Images were obtained using a Canon A620 camera on an Axiovert 40 CFL microscope (Carl Zeiss) with a Plan Neofluar 100×/NA 1.3 oil objective, using Endow GFP and Cy3 narrow-band excitation filter sets for fluorescent images. Merge images of MitoTracker and pHluorin signals were created using Photoshop software.

In situ pHluorin calibration and pHluorin measurements. Strains were grown in Erlenmeyer flasks to an OD₆₀₀ of approximately 1.0 in Verduyn medium, centrifuged for 5 min at 4000 r.p.m. and resuspended in PBS containing 100 µg digitonin ml⁻¹. After 10 min cells were washed with PBS and put on ice. Cells were transferred to CELLSTAR black polystyrene clear-bottom 96-well microtitre plates (Greiner Bio-One) to an OD₆₀₀ of 0.5 in citric acid/Na₂HPO₄ buffer of pH values ranging from 5.0 to 9.0. pHluorin fluorescence emission was measured at 512 nm using a SpectraMax Gemini XS microtitre plate spectrofluorometer (Molecular Devices) providing excitation bands of 9 nm centred around 390 and 470 nm. Background fluorescence for a wild-type culture not expressing pHluorin was subtracted from the measurements. The ratio of emission intensity resulting from excitation at 390 and 470 nm was calculated ($R_{390/470}$) and plotted against the corresponding buffer pH. In all experiments a wild-type culture was grown simultaneously as a reference for background fluorescence at both separate excitation wavelengths. pH values are always represented as means ± SD. All pH determination experiments were repeated at least three times (biological replicates) and figures show one representative experiment in which error bars represent the standard deviation of at least three technical replicates.

Perturbations. Cells were pre-cultured in Erlenmeyer flasks to an OD₆₀₀ of approximately 1.0 in Verduyn medium with 2% of the carbon source indicated. Cells were reinoculated in batch fermenters in Verduyn medium with controlled pH. For chitosan treatment this pH was 6.7. When cultures reached an OD₆₀₀ of 0.2 fragmented chitosan was added to a final concentration of 50 µg ml⁻¹. Chitosan was kindly provided by Dr A. Zakrzewska and fragmented as previously described (Zakrzewska *et al.*, 2005). For pH-shift experiments cells were reinoculated in batch fermenters at the pH indicated. At an OD₆₀₀ of 0.2, the pH was changed to the post-shift value by titration with 1 M HCl or 1 M KOH. For sorbic acid treatment cells were reinoculated in batch fermenters with a pH of 5.0. When cultures reached an OD₆₀₀ of 0.2 potassium sorbate was added in given concentrations. OD₆₀₀ and pH were registered over time. For the studies concerning carbon source and the effect of respiratory inhibitors, cells were reinoculated in shake flasks with Verduyn medium buffered at pH 5.0 with 25 mM sodium citrate, with 2% glucose, 2% galactose or 2% glycerol and 2% ethanol. At an OD₆₀₀ of 0.2, samples were transferred to microtitre plates, in the presence or absence of 10 µM antimycin A or carbonyl cyanide *m*-chlorophenylhydrazone (CCCP, Sigma).

Glucose pulse experiments. Cells were grown to an OD₆₀₀ of approximately 1.0 in shake flasks in Verduyn medium and washed twice with Verduyn medium without glucose. Subsequently, cells were inoculated in batch fermenters to an OD₆₀₀ of 0.2 in Verduyn medium without glucose at pH 5.0 and starved for at least 1 h. Glucose was administered to the batch fermenters to a final concentration of 10 mM at time point $t=0$. For short-term glucose pulse experiments, cells were taken from the batch fermenter prior to glucose addition and transferred to microtitre plates; a 10 mM glucose pulse was given in the plate. Time point $t=0$ was measured just before glucose addition.

RESULTS

pHluorin expression in cytosol and mitochondria

To differentially determine pH_{mit} and pH_{cyt} pHluorin was subcloned into multicopy plasmids either with or without a mitochondrial targeting signal. For strong, constitutive pHluorin expression we introduced the promoter region of the *ACT1* gene. The mitochondrial targeting signal consisted of the first 69 amino acids of subunit 9 of the F₀ ATPase of *Neurospora crassa*, comprising the N terminus of the pHluorin fusion protein. Localization of a GFP protein to the mitochondrial network by means of this targeting signal was previously described by Westermann & Neupert (2000). To confirm proper localization of the pHluorin in both strains, the subcellular localization of the green fluorescence emitted from the proteins was compared to that of the red fluorescence of a MitoTracker dye in cells grown on glucose (not shown) and on glycerol/ethanol to obtain maximal mitochondrial networks (Fig. 1).

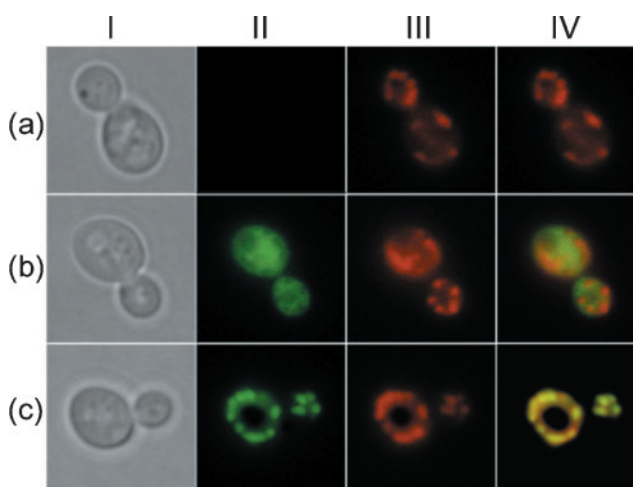


Fig. 1. Targeting of pHluorin to cytosol and mitochondria. (a) Wild-type, (b) ORY001 and (c) ORY002 strains were grown in Verduyn medium with 2% glycerol and 2% ethanol to mid-exponential phase at 30 °C. Then 25 nM MitoTracker Red CMXRos was added to the medium and cells were cultured for an additional 30 min. Images show (I) phase-contrast, (II) pHluorin and (III) MitoTracker signals. Column IV shows merged images of pHluorin and MitoTracker signals.

pHluorin expression in strain ORY001, which contained a multi-copy plasmid carrying the pHluorin construct (Table 1), was diffusely distributed over the cell (Fig. 1b). In this strain the protein was excluded from mitochondria as the green and red signals did not co-localize. Additionally, exclusion of both signals from another organelle, which probably corresponded to the vacuole, was also observed. These observations are consistent with pHluorin localization in the cytosol. Strain ORY002, containing a plasmid with the pHluorin gene preceded by a mitochondrial targeting sequence, showed pHluorin expression that clearly co-localized with the MitoTracker dye (Fig. 1c), consistent with pHluorin expression in the mitochondria.

To assess whether cellular functioning was affected by the overexpression of pHluorin in cytosol and mitochondria, we determined the physiological parameters of respiring, glucose-limited chemostat cultures of wild-type cells as well as ORY001 and ORY002 (Table 2). We found that, although expression of both cytosolic and mitochondrial pHluorin led to a small decrease in CO₂ and O₂ fluxes, it did not affect specific biomass yield, glucose flux or respiratory quotient. No significant differences were detected between the two pHluorin-expressing strains. These data show that overexpression of pHluorin in cytosol and mitochondria did not disturb cellular metabolism.

pH monitoring in cytosol and mitochondria

To obtain *in situ* calibration curves for pH measurements, cultures were grown to an OD₆₀₀ of approximately 1.0 and subjected to mild permeabilization by incubation with 100 µg digitonin ml⁻¹ for 10 min. Cells were resuspended in buffers of known pH in the range 5.0–9.0 and ratios of pHluorin emission by 390 and 470 nm excitation ($R_{390/470}$) were plotted against pH after background subtraction (Fig. 2a, b). These plots were used to calibrate our *in vivo* measurements. To confirm that we measured accurate absolute pH values, we used mild treatment with the membrane-perturbing compound chitosan (Zakrzewska *et al.*, 2005). We cultured our strains in batch fermenters at pH 6.7 (dashed line in Fig. 2c) to mid-exponential phase, and monitored unstressed pH_i values for about 30 min. This revealed a pH_{cyt} around 7.2 and a slightly higher pH_{mit} around 7.5 (Fig. 2c). After addition of chitosan to a final concentration of 50 µg ml⁻¹ at $t=0$, pH values gradually declined and equilibrated to the external pH of

6.7 within 1 h. Subsequently, we lowered the medium pH to 6.0 by addition of HCl, and after 15 min increased the pH to 7.0 using KOH (dashed line). Our pH_i measurements accurately followed these changes (Fig. 2c). We conclude from these data that the pH in both cytosol and mitochondria can be measured separately and accurately.

Starved cells pulsed with glucose show a rapid acidification followed by alkalinization

A well-known phenomenon in yeast is glucose pulse-induced initial acidification and subsequent alkalinization of glucose-starved cells (Martínez-Muñoz & Kane, 2008; Kresnowati *et al.*, 2007; Ramos *et al.*, 1989; van Urk *et al.*, 1989). While reports on the kinetics of this phenomenon are similar, the methods of culturing and pH_i determination vary greatly, probably significantly contributing to the differences in actual pH_i values reported. Glucose-starved cultures (Fig. 3b) showed pH_i values significantly lower than cultures growing on glucose-containing media (Figs 2c, 4a, b, 5a–c, 6c, d). Both pH_{cyt} and pH_{mit} values decreased to 5.7 during the starvation period, abolishing the pH difference between the two organelles. At $t=0$ glucose was added, and an alkalinization of the batch culture was observed to pH_i values transiently higher than the pH_i of glucose-grown cultures. After approximately 60 min both pH_{cyt} and pH_{mit} recovered to those of glucose-growing cultures in mid-exponential phase (Fig. 3b). At the same time, the cultures resumed growth (Fig. 3a), indicative of a relation between pH_i and growth. The initial acidification reported in earlier experiments could not be observed, which could be explained by the fact that this is a fast effect that cannot be observed on a timescale of minutes.

To be able to evaluate shorter timescales we next performed an experiment with glucose-starved cells from the fermenter using microtitre plates. After addition of glucose, we monitored the pH_i four times for 100 s with 13 s intervals. To get optimal time resolution we started each subsequent measurement series 2 s after the previous one. The data were plotted in a single figure (Fig. 3c). Equipment limitations prevented measuring the kinetics of the first 20 s. Still, with this set-up we could observe the initial acidification. A rapid and transient drop in pH_{cyt} could be observed from 5.7 to 5.3 within 20 s, followed by an immediate alkalinization to 6.5 within the first 100 s.

Table 2. Physiological parameters and pH of pHluorin-expressing strains in glucose-limited continuous cultures

Data are the means ± SD of three independent chemostat cultures for each strain. Ethanol was not detectable in these conditions. q_{Glucose} , q_{O_2} and q_{CO_2} values are expressed as mmol (g dry wt)⁻¹ h⁻¹.

Strain	Dry weight (g l ⁻¹)	Yield (g g ⁻¹)	q_{Glucose}	q_{O_2}	q_{CO_2}	pH _{cyt}
CEN.PK 113-7D	3.8 ± 0.2	0.50 ± 0.03	1.1 ± 0.1	2.9 ± 0.3	2.9 ± 0.3	ND
ORY001	3.7 ± 0.2	0.50 ± 0.05	1.1 ± 0.1	2.5 ± 0.1	2.5 ± 0.1	6.9 ± 0.1
ORY002	3.8 ± 0.2	0.54 ± 0.03	1.0 ± 0.1	2.5 ± 0.1	2.5 ± 0.1	7.3 ± 0.1

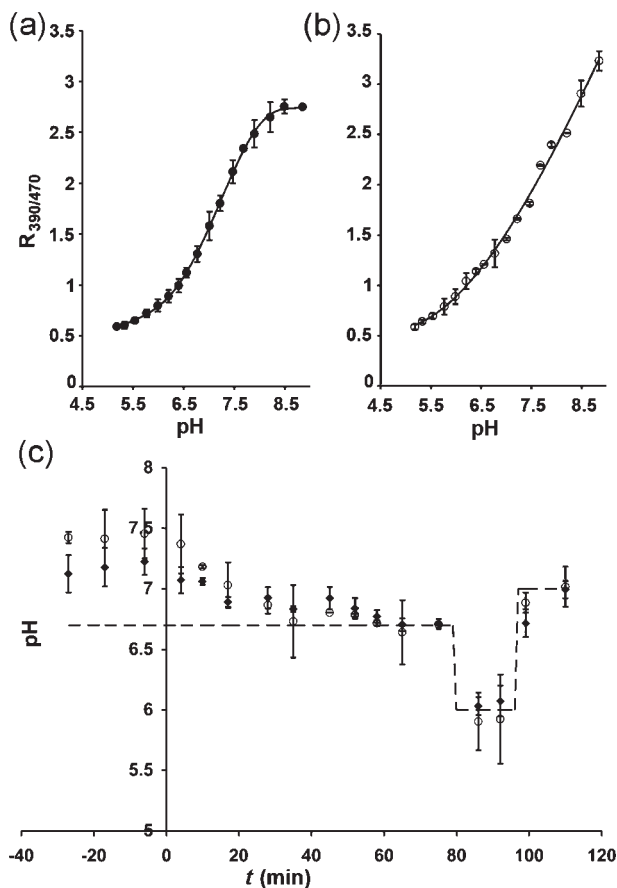


Fig. 2. Calibration curves and *in situ* determinations of pH_{cyt} and pH_{mit} . (a, b) Strains were grown in Erlenmeyer flasks to an OD_{600} of approximately 1.0 in Verduyn medium and permeabilized using $100 \mu\text{g}$ digitonin ml^{-1} . Cells were washed and resuspended in citric acid/ Na_2HPO_4 buffer of pH values ranging from 5.0 to 9.0. Ratios of pHluorin emission at 512 nm upon excitation at 390 and at 470 nm ($R_{390/470}$) were plotted against pH after wild-type background subtraction to provide calibration curves for (a) strain ORY001 and (b) strain ORY002. (c) Strains were grown to mid-exponential phase in batch fermenters in Verduyn medium at pH 6.7. At $t=0$ fragmented chitosan was added to a final concentration of $50 \mu\text{g ml}^{-1}$. At $t=80$ and $t=95$ the pH of the medium was set to 6 and 7 using HCl and KOH respectively. Error bars represent standard deviation of three technical replicates. ●, pH_{cyt} ; ○, pH_{mit} ; dashed line, medium pH.

The pH_{mit} did not show an acidification and increased to 7.2 in the first 100 s. Thus, organelle-specific expression of pHluorin allows a direct and distinctive kinetic monitoring of organellar pH.

Effect of respiratory chain inhibitors on pH_{cyt} and pH_{mit} depends on carbon source

While the pH_{cyt} measurements recapitulate previous findings, the pH_{mit} behaves differently. Also, there might be a difference in fermenting versus respiring cells. Indeed,

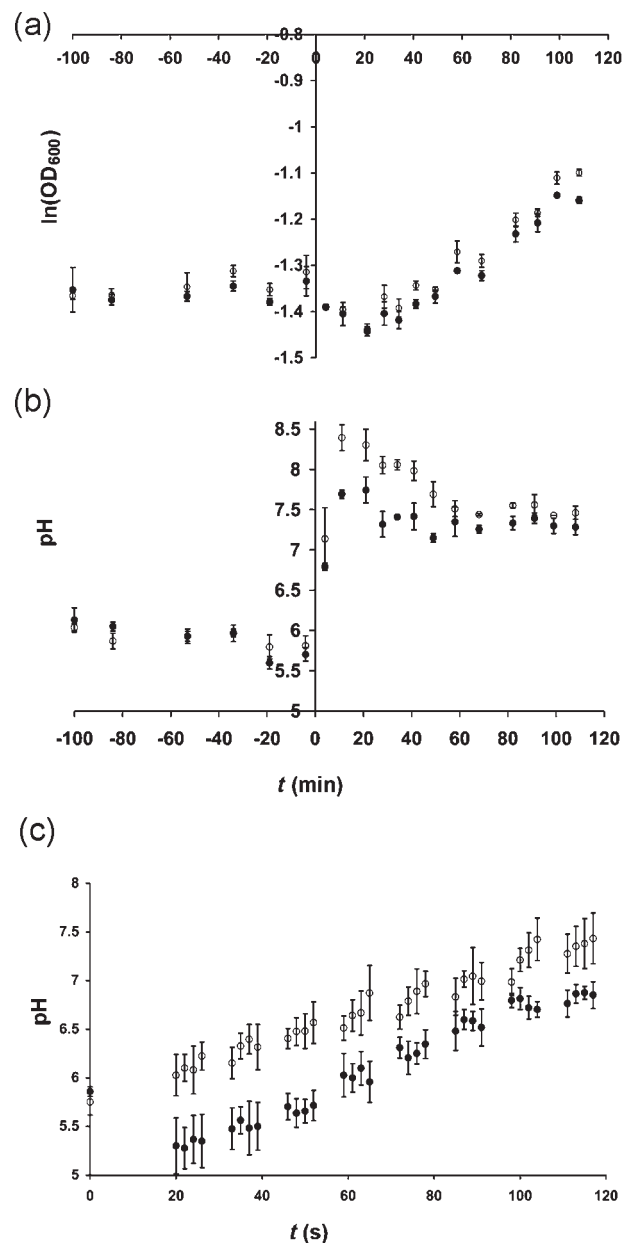


Fig. 3. Long- and short-term effects of an external glucose pulse on the pH_i of starved cells. (a, b) Cultures were starved as described in Methods and reinoculated in batch fermenters containing Verduyn medium without glucose. At $t=0$ batch cultures were pulsed with glucose to a final concentration of 10 mM. (a) Growth curves, (b) pH profiles. Error bars represent standard deviation of three technical replicates. (c) Samples of glucose-starved cells were transferred to microtitre plates followed by a glucose pulse to a final concentration of 10 mM. Time point $t=0$ was measured just before glucose addition. Error bars represent standard deviation of seven technical replicates. ●, pH_{cyt} ; ○, pH_{mit} .

in our chemostat cultures respiring glucose (Table 2) we determined a pH_{cyt} of 6.9 and a pH_{mit} of 7.3, a pH

difference already much bigger than that in glucose-fermenting batch cultures. To observe how pH_{cyt} and pH_{mit} are differentially controlled, we cultured our strains on media with different carbon sources. Glucose-containing batch cultures are fully fermenting. Galactose can be metabolized in fermentative and respiratory metabolism, and does not cause glucose repression. In galactose-grown batch cultures *S. cerevisiae* has fully functional mitochondria and respiratory activity (Lagunas, 1976). Lastly, glycerol/ethanol-containing media can only be used via respiration. On these different media, both pH_{cyt} and pH_{mit} change. In cultures grown on glucose, galactose and glycerol/ethanol, we measured pH_{cyt} values of 7.0 ± 0.0 , 7.1 ± 0.1 and 6.8 ± 0.0 , respectively, while pH_{mit} was registered at 7.5 ± 0.0 , 7.30 ± 0.1 and 7.26 ± 0.0 (see Fig. 4, $t=0$). There was no significant effect of respiratory conditions on the pH difference between mitochondria and cytosol, however. We then assessed the effect of inhibition of mitochondrial respiration on organellar pH. We stressed cultures using the respiratory chain electron transfer inhibitor antimycin A (Fig. 4a, c, e), or the proton gradient uncoupler CCCP (which affects plasma membrane proton gradient as well; Fig. 4b, d, f). Antimycin A prevents energy

generation using the respiratory chain, and causes a rapid drop in mitochondrial and pH_{cyt} in cells grown on galactose (Fig. 4c) and glycerol/ethanol (Fig. 4e), but has very little effect on glucose-grown cells (Fig. 4a), which have repressed respiratory chain expression. Interestingly, in glucose-grown cells the ΔpH over the mitochondrial membrane is maintained (Fig. 4a), while it is lost in derepressed cells (Fig. 4c) and even reversed in fully respiratory cells (Fig. 4e). With all three carbon sources, addition of CCCP leads to a complete dissipation of the pH gradient over the mitochondrial membrane. Interestingly, the pH reached after 20 min of treatment depends on the culture carbon source, where respiratory, glycerol/ethanol-grown cells seem to equilibrate with the external pH (Fig. 4f), while galactose- and glucose-grown cells can control pH_i for longer (Fig. 4b, d).

pH_i values are unaffected by external pH shifts between pH 3.0 and pH 7.5

Baker's yeast is very resistant to acidic environments, and can grow in cultures with a pH as low as 2.5 (Carmelo *et al.*, 1996). We investigated how external pH changes

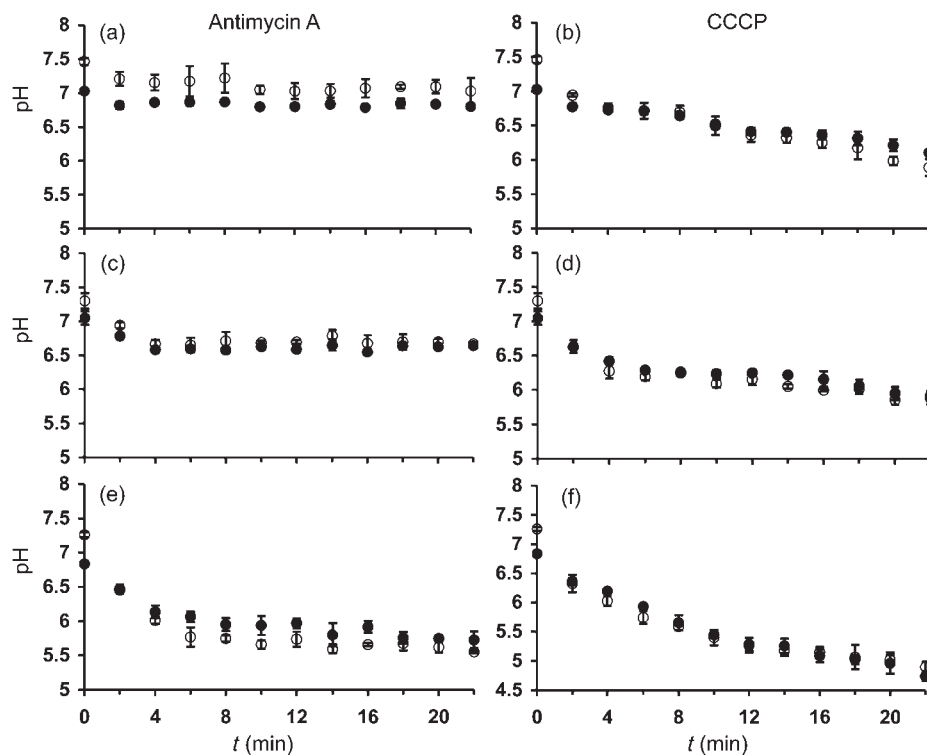


Fig. 4. Effect of respiratory inhibitors on pH_i depends on the extent of glucose repression. Cultures were grown in media containing 2% glucose (a, b), 2% galactose (c, d), or 2% glycerol and 2% ethanol (e, f) as carbon sources. (a, c, e) Effect of treatment with 10 μM antimycin A on pH_{cyt} (●) and pH_{mit} (○) on cultures grown to exponential phase on (a) glucose, (c) galactose, and (e) glycerol/ethanol. (b, d, f) Effect of treatment with 10 μM CCCP on pH_{cyt} (●) and pH_{mit} (○) on cultures grown to exponential phase on (b) glucose, (d) galactose, and (f) glycerol/ethanol. Error bars represent standard deviation of four technical replicates.

influence the pH_i using our pHluorin-based method. As protons are charged we do not expect them to diffuse over the plasma membrane. However, changes in the pH of the medium affect the pH gradient over the plasma membrane, which affects ATPase activity (Carmelo *et al.*, 1996), and previous reports have described the dependency of pH_i on the extracellular pH (Valli *et al.*, 2005). To investigate if pH_i values were sensitive to changes in the external proton concentration, we shifted the pH of the medium of cells grown at pH 6.0 to pH 3.0 using HCl (Fig. 5a), and the pH of the medium of cells grown at pH 3.0 to pH 6.0 using

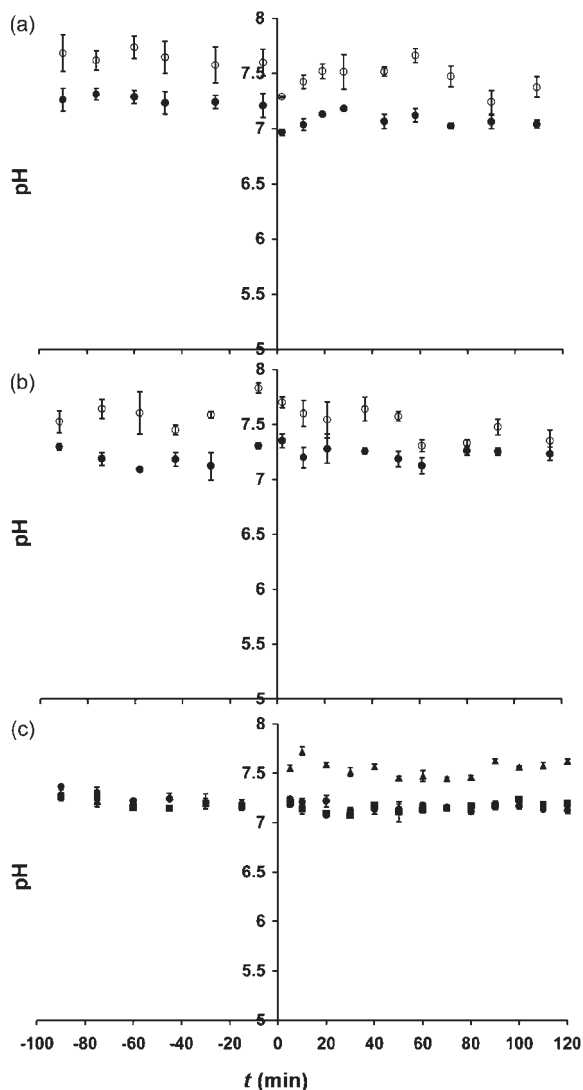


Fig. 5. pH_i values are unaffected by external pH between pH 3.0 and pH 7.5. (a, b) Strains were grown to mid-exponential phase in batch fermenters in Verduyn medium at pH 6.0 (a) or pH 3.0 (b). At an OD_{600} of 0.2 ($t=0$) the pH was rapidly titrated to pH 3.0 using HCl (a) or to pH 6.0 using KOH (b). ●, pH_{cyt} ; ○, pH_{mit} . (c) Cultures were grown at pH 6.0 and rapidly titrated to pH 7.0 (●), pH 7.5 (■) or pH 8.0 (▲) using KOH. Error bars represent standard deviation of three technical replicates.

KOH (Fig. 5b). All cultures showed a doubling time of 109 min and the shifts in external pH did not affect the growth rates of the cultures (unpublished data). Fig. 5(a) shows that the effect of a sudden acidification on pH_{cyt} and pH_{mit} is negligible on a timescale of minutes. A similar absence of response in pH profiles was observed when cultures were subjected to a sudden alkalization (Fig. 5b). Additionally, we measured pH_{cyt} in cultures shifted from pH 6.0 to pH values between 7.0 and 8.0 (Fig. 5c). Shifts to pH 7.0 and 7.5 did not lead to a change in pH_i . However, shifting to an external pH of 8.0, much higher than the stable internal pH of 7.2, did lead to decreased growth, and to an increase in pH_i to a new (stable) value of 7.5. However, these cultures showed evidence of cell lysis; the cultures developed foam, and when we analysed the culture supernatant we detected fluorescence at pHluorin wavelengths, which we did not detect in supernatants from other cultures (unpublished data), suggesting leakage of possibly 50% of the pHluorin out of the cell. The pH_i values determined are therefore unreliable, as they result from an average fluorescence of pHluorin in the culture supernatant and pHluorin in the cell. We conclude that external shifts in proton concentration in the pH range from 3.0 to 7.5 do not significantly affect pH_i values. High pH values, causing a reversal of the pH gradient, caused cell lysis.

pH_i dynamics during sorbic acid stress and recovery coincide with the effects observed on growth

Weak organic acids are presumed to cause pH_i homeostasis stress (Bracey *et al.*, 1997; Brett *et al.*, 2005). The undissociated, uncharged molecule is thought to diffuse over the plasma membrane and dissociate at the higher pH encountered in the cell, which leads to acidification as well as anion accumulation (Piper *et al.*, 2001). We assessed the effect of the food preservative sorbic acid on growth and pH_i . We used concentrations of 2.74 and 5.47 mM potassium sorbate, which at pH 5.0 corresponds to 1 and 2 mM undissociated sorbic acid respectively. Cultures maintained full viability under these stress conditions (unpublished data). The presence of 1 mM sorbic acid resulted in a reduction of exponential growth rate of 50–60% in both strains, whereas 2 mM resulted in a nearly complete growth stasis (>90% inhibition) within the time-frame studied (Fig. 6a, b).

Similar to our previous experiments, cells in unstressed glucose-growing cultures maintained a pH_{cyt} of 7.2 and a pH_{mit} of 7.5 (Fig. 6c, d). When cultures were stressed with 1 mM sorbic acid, an immediate decrease in pH_{cyt} to 6.2 could be observed, which, after a lag of approximately 1 h, increased to a value of 6.7 over the next 2 h and then stabilized (Fig. 6c). When cells were stressed with 2 mM sorbic acid, pH_{cyt} dropped to 6.0 and did not increase for at least 4 h. Similarly, the pH_{mit} decreased from 7.5 to 6.6 when cultures were stressed with 1 mM sorbic acid, and

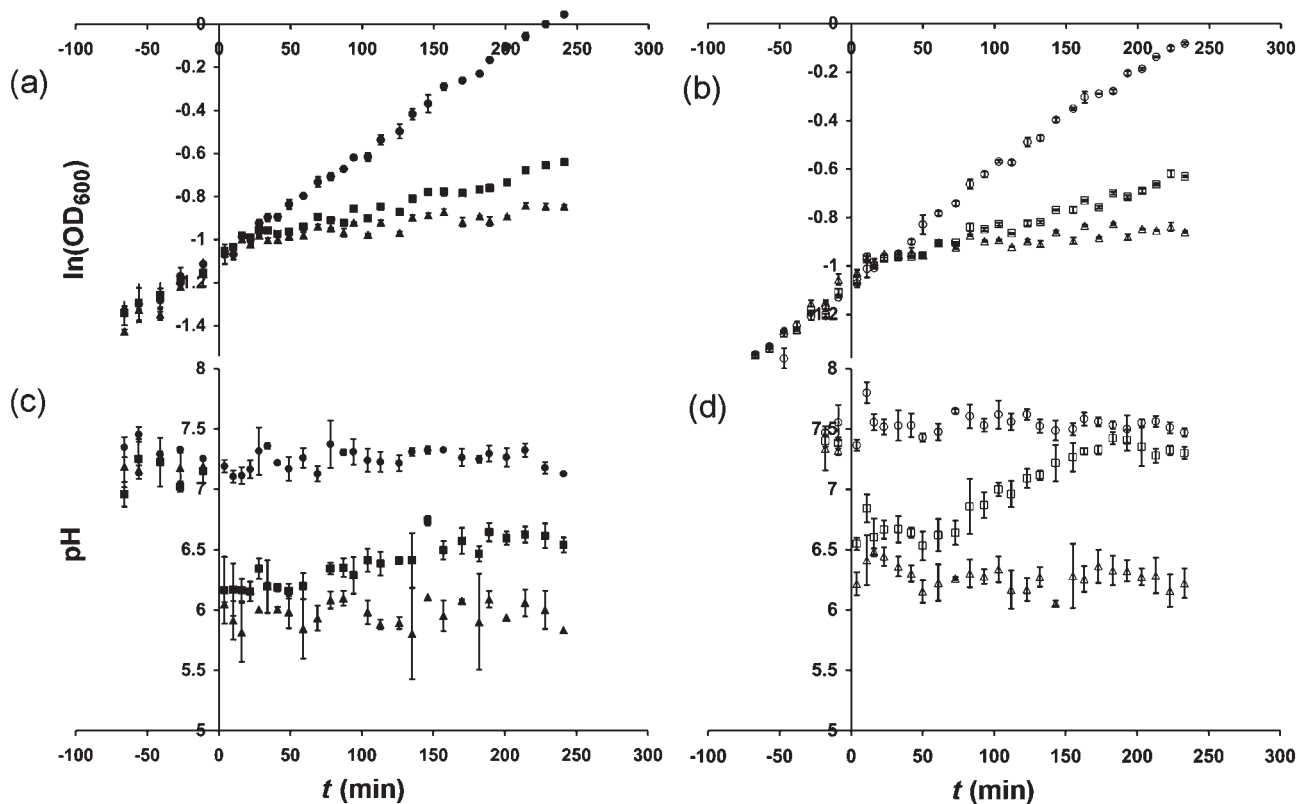


Fig. 6. Effects of sorbic acid on pH_i . Strains were grown to mid-exponential phase in batch fermenters in Verduyn medium at pH 5.0. Filled symbols, ORY001/ pH_{cyt} ; open symbols, ORY002/ pH_{mit} . At an OD_{600} of 0.34 potassium sorbate was added ($t=0$) to final concentrations of 0 mM (●, ○), 2.74 mM (■, □) and 5.74 mM (▲, △). (a) Growth curve of ORY001, (b) growth curve of ORY002, (c) pH_{cyt} profiles, (d) pH_{mit} profiles. Error bars represent standard deviation of three technical replicates.

recovered to a pH of 7.3 after a lag of 1 h and a recovery period of 90 min (Fig. 6d). When cultures were stressed with 2 mM sorbic acid, pH_{mit} dropped to 6.3 and, like pH_{cyt} , did not recover for at least 4 h.

Comparing these results to the growth curves we observed that cells stressed with 1 mM sorbic acid were also able to recover growth. These cultures partly recovered their pH_i values (Fig. 6c, d) and showed a reduced growth rate of approximately 60% of their original growth rate (Fig. 6a, b). Cells stressed with 2 mM sorbic acid did not recover their pH_i and showed no significant growth recovery. Interestingly, the ΔpH between cytosol and mitochondria was maintained during sorbic acid stress and even increased in cultures stressed with 1 mM sorbic acid. In these cells ΔpH was as large as 0.6 pH units after 3 h of stress as opposed to 0.3 in unstressed cultures (Fig. 6c, d). This suggests that cells may be able to at least maintain a level of oxidative phosphorylation under this level of sorbic acid stress. We conclude that pH_i dynamics during sorbic acid stress and recovery coincide with the effects observed on growth. Whether pH_i is causal with respect to growth rate, or simply generally associated with decreased growth rates, cannot yet be concluded.

DISCUSSION

The pH inside a compartment of any living cell is an important parameter. Changes in pH influence the ionization state of all weak acids and weak bases, which includes all peptides and proteins. Cellular processes that are affected by diverging pH values include transport of molecules over membranes (Nishimura *et al.*, 1998) and key metabolic processes such as redox state (Veine *et al.*, 1998). Not surprisingly, most organisms try to regulate the pH in specific organelles (Martínez-Muñoz & Kane, 2008; Llopis *et al.*, 1998; Roos & Boron, 1981). We set out to accurately determine the organelle-specific pH in live, unperturbed yeast cells. We therefore expressed ratiometric pHluorin (Miesenböck *et al.*, 1998) in the cytosol and the mitochondrial matrix. This yielded fast (seconds timescale) and accurate pH measurements in cytosol and mitochondria. The range of pH values that can be accurately measured is limited, as the resolution of the calibration curve deteriorates below 5.5 and above 8.0, but it is sufficient to measure pH values currently reported in living cells (Brett *et al.*, 2006). We determined a pH_{cyt} of 7.2 ± 0.2 and a pH_{mit} of 7.5 ± 0.2 in cells growing on glucose. These specific pH values appear to be tightly regulated and

maintained in a broad range of external pH values, ranging from pH 3.0 to 7.5 (Figs 2c, 5a–c and 6). Previously reported pH_i values were lower (Bracey *et al.*, 1998; Fernandes *et al.*, 2003; Guldfeldt & Arneborg, 1998). This is most likely due to the methods used, which determine an average pH over whole cells including the vacuole. This will result in significantly lower values. Indeed, others using pHluorin report similar pH_{cyt} values (Martínez-Muñoz & Kane, 2008; Brett *et al.*, 2005). We have not determined vacuolar pH, even though this organelle is important for cellular pH homeostasis and is crucial in the weak acid stress response (Martínez-Muñoz & Kane, 2008; Makrantoní *et al.*, 2007; Piper *et al.*, 2001). pHluorin could not be used to report on vacuolar pH, because of both the reduced reliability of the reporter in the expected low-pH environment (Martínez-Muñoz & Kane, 2008; Brett *et al.*, 2005; Smith *et al.*, 2002) and the apparent instability of the pHluorin targeted to the vacuole (our unpublished data). Possibly other fluorescent proteins with lower pK_a values or different maturation requirements will yield successful tools in the future (Shaner *et al.*, 2005; van Roermund *et al.*, 2004).

Effect of external pH on pH_i

There are several reports in the literature on the transcriptional and physiological effects of a shift of the extracellular pH to low pH (Causton *et al.*, 2001; Kapteyn *et al.*, 2001; Motizuki *et al.*, 2008). External pH was also shown to affect pH_i when pH_i was assayed in buffers instead of media (Martínez-Muñoz & Kane, 2008). Remarkably, our experiments show that a medium pH of 3.0 does not lead to a significant change of pH_{cyt} or pH_{mit} (Fig. 5) and also has no apparent effect on growth. Two aspects contribute to this discrepancy. First, usually complex media which contain (unwanted) weak acids are used, or weak-acid buffers are added to the medium. Lowering the pH in these media causes protonation of these compounds, and thus induces a weak-acid stress that lowers pH_i . Evidence of this is provided by our observation that a shift in pH from 6.0 to 4.2 in complex YPD medium results in a decrease in growth rate, with doubling times increasing from 77 to 88 min (our unpublished data), which is in marked contrast to the absence of any effect on growth rate upon a shift from pH 6.0 to 3.0 in defined medium. We conclude that extracellular pH does not affect pH_i in growing cells, except when it is much higher than pH_i , as evidenced by the cell lysis at pH 8.0 (see Fig. 5).

Effect of nutrient availability on pH_i

pH_{cyt} was reported to decrease transiently and then increase upon addition of glucose to glucose-limited or starved cells (Martínez-Muñoz & Kane, 2008; Kresnowati *et al.*, 2007; Ramos *et al.*, 1989; van Urk *et al.*, 1989). We observed the same (Fig. 3). The availability of nutrients also appears crucial for maintaining the pH gradients over organellar membranes, as exemplified by the difference

between cytosolic and vacuolar pH values assayed in buffers compared with those measured in cells growing in defined media (Martínez-Muñoz & Kane, 2008; Brett *et al.*, 2005), as well as the dissipation of the ΔpH over the mitochondrial membrane in glucose-starved cells (Fig. 3).

We generated a tool that allows an easy assessment of the pH gradient over the mitochondrial membrane, which is a crucial part of the energy-generating proton-motive force. Thus, we could determine that the proton ionophore CCCP led to a dissipation of the ΔpH over the mitochondrial membrane within minutes, irrespective of the carbon source used for cultivation. Interestingly, the equilibration of pH_i with external pH, also expected to occur in CCCP-treated cells, did depend on the culture carbon source, which may be caused by the absence of any means of energy generation in glycerol/ethanol-grown cells without a proton-motive force. Blocking of electron transfer from ubiquinol to cytochrome *b/c*₁ using antimycin A hardly had any effect on glucose-grown cells, which do not depend on a highly functional respiratory chain (Lagunas, 1976). In galactose-grown cells the gradient did dissipate, while it was even reversed in glycerol/ethanol-grown cells (Fig. 4). This suggests that in glucose-repressed cells the ΔpH , still important for uptake of compounds and import of proteins into the mitochondria, can be maintained independent of respiratory chain activity. Interestingly, again we see that pH_{cyt} is affected most in glycerol/ethanol-grown cells, and least in glucose-grown cells, reflecting the need for energy-generating capacity in maintaining a neutral pH_{cyt} .

We hypothesize that cytosolic acidification in cells grown on galactose or glycerol/ethanol, but also in glucose-starved cells (Fig. 3), is caused by protons that enter the cytosol during the uptake of various nutrients, for which the plasma membrane proton gradient is the driving force (Goffeau & Slayman, 1981; Van Leeuwen *et al.*, 1992). This acidification would normally be counteracted by V-ATPases pumping protons into the vacuole (Martínez-Muñoz & Kane, 2008) or the plasma membrane ATPase, which translocates protons out of the cytosol into the medium, both at the cost of ATP, to keep the pH_{cyt} constant. A shortage of energy as a result of carbon starvation or respiratory chain inhibition would abolish the activity of the plasma membrane ATPase, resulting in a decrease in pH_i , ultimately to the pH of the environment (Karagiannis & Young, 2001). A decrease of pH_{cyt} also affects the redox balance, shifting the NADH/NAD⁺ equilibrium to the oxidized form and thus lowering the cytosolic NADH concentration (Veine *et al.*, 1998). Additionally, glucose starvation results in a severely reduced glycolytic flux, which also leads to a decrease in the cytosolic NADH concentration (Vemuri *et al.*, 2007). This decrease of NADH in its turn leads to a reduced translocation of protons over the mitochondrial matrix membrane. Ultimately, pH_{mit} will also equilibrate to the pH of the environment.

After the addition of glucose, pH_i values were restored to values observed in growing cells (Fig. 3b) and cells resumed growth (Fig. 3a). Similarly, sorbic acid-stressed cells recovered their pH_i when they resumed growth, whereas cells treated with concentrations of sorbic acid that caused a permanent growth stasis also did not show pH_i recovery (Fig. 6) This raises the question whether the lowered pH_i is actually causing the reduction in growth rate observed in the presence of sorbic acid. Interestingly, although both pH_{cyt} and pH_{mit} are lowered, the weak-acid stress does not collapse the ΔpH over the mitochondrial inner membrane. How this gradient is linked to energy generation and cellular growth under these conditions remains to be elucidated.

Concluding remarks

We have shown that there is a remarkable coincidence between pH_i , energy-generating capacity and growth rate. Our toolset will allow us to generate a deeper understanding of how pH_i affects cellular metabolism and growth, and vice versa.

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