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Cyanohydrin reactions enhance glycolytic oscillations in yeast

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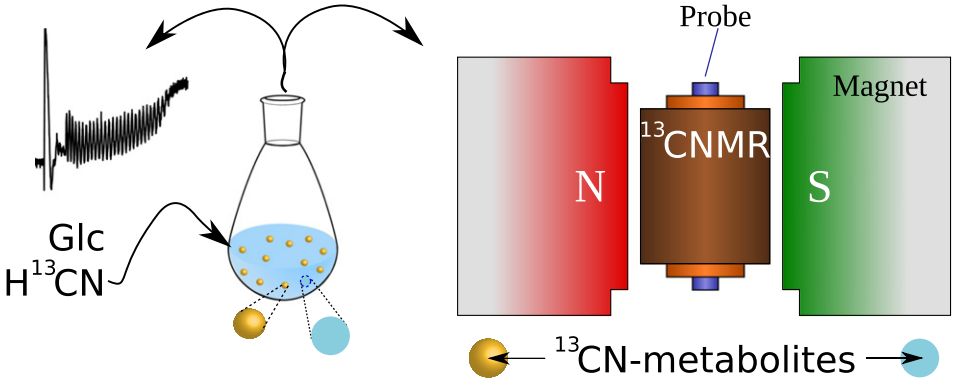
HIGHLIGHTS

- Fermenting yeast produce intracellular cyanohydrins if fed with cyanide.
- Yet, extracellular lactonitrile production accounts for ~66% of cyanide consumption.
- During fermentation, cyanide mainly reacts with intracellular carbonyl compounds.
- Intracellular cyanide reactions augment the amplitude of oscillations.

GRAPHICAL ABSTRACT



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ABSTRACT

Synchronous metabolic oscillations can be induced in yeast by addition of glucose and removal of extracellular acetaldehyde (ACA_x). Compared to other means of ACA_x removal, cyanide robustly induces oscillations, indicating additional cyanide reactions besides ACA to lactonitrile conversion. Here, ^{13}C NMR is used to confirm our previous hypothesis, that cyanide directly affects glycolytic fluxes through reaction with carbonyl-containing compounds. Intracellularly, at least 3 cyanohydrins were identified. Extracellularly, all signals could be identified and lactonitrile was found to account for ~66% of total cyanide removal. Simulations of our updated computational model show that intracellular cyanide reactions increase the amplitude of oscillations and that cyanide addition lowers [ACA] instantaneously. We conclude that cyanide provides the following means of inducing global oscillations: a) by reducing [ACA_x] relative to oscillation amplitude, b) by targeting multiple intracellular carbonyl compounds during fermentation, and c) by acting as a phase resetting stimulus.

1. Introduction

In a stirred suspension of yeast cells harvested at the diauxic shift, synchronous metabolic oscillations can be induced by addition of glucose and addition of cyanide [1,2]. The mechanism of yeast cell communication and synchronization is still an unresolved question. Extracellular acetaldehyde (ACA_x) seems to be the (main) synchronizing agent [3–5] and hence the synchronization signal depends on the ratio between the oscillatory amplitude and average concentration of ACA_x. However, direct dynamic evidence for [ACA_x]-oscillations in the extracellular medium remains elusive. Removal of ACA_x using e.g. $N_{2(g)}$ has been shown to induce oscillations [6,7]. However, a much stronger propensity to induce oscillations is observed using cyanide [6]. Recently, we found that much cyanide is consumed during fermentation, hypothesizing that cyanide not only reacts with ACA_x, but also

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with unidentified intracellular carbonyl compounds [8]. It is well-known that glucose (Glc), fructose (Fru), pyruvate (Pyr), dihydroxyacetone-phosphate (DHAP), and ACA are the major carbonyl compounds produced during fermentation [9]. Using ^{13}C-NMR, we show that Pyr, DHAP, ACA, and smaller sugars readily react with cyanide, while the hexoses only show weak to no reaction in the same reaction conditions used under fermentation. The obtained chemical shifts of these cyanohydrins are used to analyze fermentation of glucose and cyanide. Extracellularly, a single cyanohydrin signal from lacto (lactonitrile) is dominant. The well-defined signal allowed for a quantitative estimate of [lacto] during fermentation. Intracellularly, the presence of at least three intracellular cyanohydrins, most likely from Pyr and intermediary sugars, was confirmed. The data obtained was used to update a computational model which subsequently was analyzed with respect to the ability of cyanide to induce oscillations. Collectively, we show that cyanide predominantly reacts with ACA_x, but also with intracellular carbonyl compounds produced during fermentation. Both modes of reaction increase the amplitude of oscillations. Finally, addition of cyanide may act as a synchronization stimulus, facilitating global oscillations. Why most cyanide reacts with ACA_x, remains however an open question.

2. Materials and methods

2.1. Cell preparation

Yeast cells (*Saccharomyces cerevisiae* X2180) were grown aerobically at 30 °C in a rotary shaker (closed batch culture) to the point of Glc depletion [10]. Harvested cells were washed twice in a 0.1 M potassium phosphate buffer (PBS), pH 6.8, followed by resuspension to the desired cell density. Cell density was determined by optical density (OD), and OD = 20 corresponding to a dry weight of 12.8 mg/ml was chosen. Finally, the cells were starved at 30 °C for 2 h and kept at ~0 °C until start of experiments.

2.2. "Close to fermentation conditions"

All experiments were carried out in conditions that resembled the fermentation conditions as closely as possible. Thus, in all experiments we used 0.1 M PBS at 25 °C and pH 7.2. Unless otherwise indicated, we only used concentrations of cyanide and metabolites that resembled the values found during fermentation (i.e. [HCN] = 5 mM and [metabolite] = 20–30 mM).

2.3. UV-Spectrometry

All spectra were measured using a Perkin-Elmer Lambda 1050 spectrometer fitted with a thermostat (25 °C) and magnetic stirring. Optical spectrometry was performed on pure substances dissolved in 0.1 M PBS and spectra from 200–800 nm were obtained from each substance. Maximal absorbance of ACA and Pyr were found at 278 nm and 320 nm, respectively. HCN did not absorb in these regions. The relative absorptivities were found to be $(0.0075 \pm 0.0006)\ \mathrm{mM^{-1}cm^{-1}}$ for ACA and $(0.0202 \pm 0.001)\ \mathrm{mM^{-1}cm^{-1}}$ for Pyr. In 0.1 M PBS these absorbances were not significantly sensitive to small reaction mediated changes in pH. Cyanohydrins derived from ACA and Pyr showed only little absorbance, but were taken into account. From the kinetic measures of HCN + Pyr ⇌ PyrCN and HCN + ACA ⇌ lacto, the rate constants were determined.

2.4. ^{13}C-NMR

All NMR spectra were measured on a 500 MHz (with a ^{13}C-cryoprobe) instrument. A sealed capillary tube containing DMSO-*d*6 was used in each NMR-tube as the reference for all chemical shift values and as a lock. Field shimming was performed before recording. During fermentation, spectra were obtained using a regular ^{1}H-decoupled pulse with a delay between scans of 2 s and 64 scans per spectra (NS = 64). This setting yielded the shortest acquisition time with consistent areas of signals and provided 9 spectra per fermentation. In experiments where the yeast cell suspension did not ferment glucose, signal-to-noise ratio was improved by increasing NS as indicated. Before these recordings, the extracellular medium was separated from the packed cells by centrifugation (18,000*g*) and cells were washed twice in 0.1 M PBS.

2.5. Analyzing spectra

Free induction decays (FIDs) were analyzed in MestReNova, version 8.1.0 (Mestrelab research S.L. 2012, Santiago de Compostela, Spain). Baseline artifacts produced by the cryoprobe were resolved and spectra were manually phase corrected.

2.6. Internal standard

MeOH was selected as 1) it gives a single signal with a chemical shift at 48.10 ppm at pH 6.8, it is separated from the other signals in the spectra from fermentation, and it remains in solution. An appropriate signal size of MeOH relative to other signal sizes was found at $c(\mathrm{MeOH}) = 22.24$ mM. For consistency a modified 0.1 M PBS buffer containing this $c(\mathrm{MeOH})$ (in the following denoted MPBS) was therefore used in all NMR experiments. This is paramount for the quantification process. The 0.1 M PBS is necessary to maintain a physiologically relevant osmolarity and to control pH in the extracellular environment. Large fluctuations in pH renders the chemical surroundings undefined, possibly obscuring identification. Importantly, we tested that MeOH did not interfere with the reactions of fermentation. First, the added amount of MeOH did not alter glycolytic oscillations. Second, using dioxane as a second internal standard, we tested that the ratio of MeOH and dioxane integrals did not change significantly during a fermentation (see section S2 in the SI).

2.7. Quantification

First, integration of a signal (here ^{13}C-lacto) was done by fitting a Lorentzian function to the signal: $f(\delta) = \frac{h\gamma^2}{(\delta-\delta_0)^2+\gamma^2}$, where δ_0 is the center of the signal, h is the height of the signal, and γ is a scale parameter specifying the half-width at half-maximum. This reduced the influence of noise. The integral of the fitted Lorentzian was used as the area of the signal. The area of signal was then related to the area of MeOH by calculating the ratio: $R = \frac{A(\mathrm{signal})}{A(\mathrm{MeOH})}$, where A is the area of the signal. Calculated R-values were used to construct a standard curve and to find concentrations of a species during yeast fermentation.

The standard curve for ^{13}C-lacto was constructed straightforwardly by a series of reactions between 50 mM H^{13}CN and ACA, assuming complete reaction and hence, at equilibration $[\mathrm{ACA}]_0 = [^{13}\text{C-lacto}]$. The ^{13}C-lacto concentration, was plotted against the experimentally determined R. From a linear regression of $[^{13}\text{C-lacto}] = a \cdot R$, the specific a for the particular NMR run-settings was used calculate [^{13}C-lacto] during fermentation.

2.8. Modeling

Building upon previous models of transient glycolytic oscillations [11,8,12], an updated model was manually fitted to capture the new NMR data. The model was simulated on a standard PC using the CVODE solver for stiff ODE systems (SUNDIALS) with a relative tolerance of 10^{-10} and absolute tolerance of 10^{-18}.