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Combined Rapid Injection NMR and Simulation Approach to Probe Redox-Dependent Pathway Control in Living Cells

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ABSTRACT Dynamic response of intracellular reaction cascades to changing environments is a hallmark of living systems. As metabolism is complex, mechanistic models have gained popularity for describing the dynamic response of cellular metabolism and for identifying target genes for engineering. At the same time, the detailed tracking of transient metabolism in living cells on the sub-minute timescale has become amenable using dynamic nuclear polarization enhanced ^{13}C NMR. Here, we suggest an approach combining in-cell NMR spectroscopy with perturbation experiments and modeling to obtain evidence that the bottlenecks of yeast glycolysis depend on intracellular redox state. In pre-steady state glycolysis, pathway bottlenecks shift from downstream to upstream reactions within few seconds, consistent with a rapid decline in the NAD^+/NADH ratio. Simulations using mechanistic models reproduce the experimentally observed response and help identify unforeseen biochemical events. Remaining inaccuracies in the computational models can be identified experimentally. The combined use of rapid injection NMR spectroscopy and in silico simulations provides a promising method for characterizing cellular reactions with increasing mechanistic detail.

INTRODUCTION

Functional behavior in living systems emerges from dynamic concentration changes of the components.¹ Such correlation between cellular functioning and reaction dynamics is also witnessed by the observation that perturbations in cellular function, for instance in disease or through engineering, are accompanied by measurable metabolic changes.² Albeit metabolism is highly conserved and central to cellular function, the current understanding of how metabolic rates are regulated arguably remains sparse.³ Such regulation occurs on a wide range of timescales, including fast timescales where effects of protein biosynthesis can be excluded.⁴ Self-regulation by metabolite (especially substrate) concentrations was previously suggested as a central pillar for maintaining a functional metabolism by changing pathway dynamics on the seconds time scale.^{3,4} Experimental approaches to noninvasively follow intracellular metabolic dynamics on this timescale are not well-established and would ideally rely on means to follow reactions of interest with natural substrates and sub-second time resolution.⁵

Due to the complexity of metabolism and due to the lack of routine experimental tools, metabolic models are often employed to translate biological systems into a system of

equations¹ to accomplish mechanistic explanations of metabolic dynamics.⁶⁻⁹ Most metabolic studies are based on the kinetics of purified enzymes in vitro. In the absence of counterpart experimental data, mechanistic models remain hard to probe for their predictive value in vivo, for instance in the understanding and design of cell factories¹⁰ or in disease intervention. Hence, a limitation for pathway modeling in systems biology has been the access to high-quality experimental in vivo data.² While it is problematic to draw conclusions on cellular functions solely from steady-state metabolite concentration measurements, kinetic measurements on cell extracts are challenged by the high turnover rate of some metabolites, requiring highly efficient quenching.² Inter-laboratory comparisons have thus highlighted the difficulty to reliably measure metabolite concentrations due to errors in inactivation, extraction or analysis.^{2,4,11}

Recently, the tracking of reaction of pathways with resolved signals from the different pathway chemicals and with sub-second time resolution has become amenable in living cells.^{5,12-18} NMR spectroscopy can experimentally provide resolved signals and the identity of the different chemicals in multistep reaction cascades. A variant of NMR spectroscopy combines chemical labelling with the NMR-visible ^{13}C isotope and a physical labelling approach that redistributes (“hyperpolarizes”)¹² nuclear spin states to achieve a short-lived boost of

NMR sensitivity. The ^{13}C -labelled hyperpolarized probe molecule improves signals of interest relative to the natural abundance background by millionfold (10,000fold through hyperpolarization and 100fold through ^{13}C isotope enrichment) and makes the detection of reactions on the sub-minute time scale feasible with a sub-second time resolution between individual spectra. Hence, this approach should permit intracellular observations of metabolic regulation on the seconds time scale.

Here, we suggest to combine hyperpolarized *in vivo* NMR spectroscopy with established experimental perturbation models and with computational simulations using mechanistic models¹⁹⁻²¹ to unravel the impact of redox-effects on glycolytic dynamics in living *S. cerevisiae*, a paradigm for studies of glycolysis and its regulation.²² Redox perturbation occurs in industrial and natural environments¹⁰ and has been implicated in the longevity of *S. cerevisiae* mother cells, when carbon limitation increases NAD^+/NADH levels.²³ Experimentally, redox perturbation can be induced with small molecules that diffuse freely and rapidly across cellular membranes.¹⁹⁻²¹ Such perturbation should affect glycolytic dynamics, as witnessed by the long known observation that oscillations in cellular redox charge accompany glycolytic oscillations.²⁴ Cellular redox state rapidly changes in pre-steady state glycolysis and, more generally, when a pulse of glucose is applied to cultured cells (Scheme 1A).^{19, 20} Altered glycolytic dynamics as a consequence of this redox change could then become observable using hyperpolarized ^{13}C NMR, once the effects of redox state on glycolytic dynamics have been defined by perturbation experiments (Scheme 1). We compared experimental observations to unbiased simulation of the experiments. Bottlenecks moved upwards in the pathway within few seconds due to rapid perturbation of cofactor pools. The approach of Scheme 1 thus provides a means to detecting redox-mediated self-regulation in living cells that contributes to the control of glycolytic fluxes in dynamically changing environments.

EXPERIMENTAL SECTION

Cell growth

Shake flask cultures of *S. cerevisiae* BY4743 were inoculated in 250 ml Erlenmeyer flasks containing 25 mL YPD medium (yeast extract (1% w/v), peptone (2% w/v), glucose (2 % w/v)) from YPD agar plates and incubated overnight under shaking at 200 rpm at 30 °C. These overnight precultures were used to inoculate the main yeast cultures of 100 mL YPD (in one-liter Erlenmeyer flasks) at a dilution of 1:100, resulting in an $\text{OD}^{600} = 0.15\text{--}0.20$. The shake flask cultures were grown at 30 °C and 200 rpm shaking to $\text{OD}^{600} = 0.8$. The cultures were harvested by centrifugation in 50 mL Falcon tubes (5000 g, 5 min) and the cell pellet was washed with 20 mL MES buffer (30 mM, pH 5.65). After a second centrifugation the pellet was resuspended in 2.2 mL fresh MES buffer (30 mM, pH 5.65) prior to transfer to a 10 mm NMR tube. Cell suspensions were starved in the absence of added glucose or other carbon source for a total of 20 minutes prior to the *in vivo* NMR experiment. For the *in vivo* NMR experiment, cell suspensions were placed in a 600 MHz Bruker spectrometer 20 min after the last resuspension.

Dynamic nuclear polarization of hexoses

Isotope-enriched metabolic substrates [$\text{U-}^{13}\text{C}$, $\text{U-}^2\text{H}$]-glucose, [$2\text{-}^{13}\text{C}$]-fructose and [$6\text{-}^{13}\text{C}, 6'\text{-}^2\text{H}_2$]-glucose were purchased from CorTec (Voisins-Le-Bretonneux, France), Cambridge Isotope Laboratories (Tewksbury, MA, USA) and Medical Isotopes Inc. (Pelham, NH, USA). All other chemicals were purchased from Sigma Aldrich (Andover, MA,

USA). Hyperpolarization was performed by solid state dynamic nuclear polarization of all hexose substrates as previously described using trityl radical OX063 (27 mM; Oxford Instruments, Abingdon, UK) and trimeric gadolinium chelate of 1,3,5-tris-(N-(DO3A-acetamido)-N-methyl-4-amino-2-methylphenyl)-[1,3,5]triazine-2,4,6-trione (0.9 mM; GE Healthcare).^{14, 25} Samples contained 90 μmol hexose in 19 mg of aqueous polarization medium containing 27 mM trityl radical OX063 and 0.9 mM trimeric Gd chelate. Samples were flash-frozen in liquid helium and polarization transfer was conducted for one hour at 1.2 K by microwave irradiation at 93.89 GHz with 100 mW using a custom-built GE Healthcare prototype polarizer with a magnetic field of 3.35 T. The solid-state polarization buildup was monitored every 5 minutes and solid-state polarizations on the order of 30% were obtained in the self-glassing carbohydrate syrup. After 1 hour of polarization, the samples were dissolved with heated Milli-Q water (4.5 mL containing EDTA (100 mg/L)) to yield liquid samples with a final substrate concentration of 20 mM hexose. Hyperpolarized substrates (600 μL) were

Scheme 1. Schematic overview of the approach taken to probe redox effect in regulating glycolytic dynamics. (A) Starvation and glucose pulse affect intracellular redox state, with possible impact on pre-steady state glycolytic dynamics if NAD^+ becomes limiting. (B) Pathway bottlenecks at high and low NAD^+/NADH ratios were emulated by perturbation with acetaldehyde and ethanol, respectively. (C) Experimental design to track pre-steady state glycolytic dynamics using a bolus of hyperpolarized glucose probe in starved cells. (D) Mechanistic details of redox effects on glycolytic dynamics were validated through a computational simulation of the experiment.

manually injected into 2.4 mL of the cell suspension placed inside a 600 MHz Bruker spectrometer. This preparation resulted in a final concentration of 4 mM hyperpolarized carbohydrate substrate for the in-cell NMR experiment.

In vivo NMR

All spectra were recorded on a DRX600 NMR spectrometer (Bruker, Karlsruhe, Germany) equipped with a 10 mm BBO probe head that was thermally equilibrated to 30 °C. The glycolytic reaction pathway was followed by a series of ^{13}C NMR spectra that were recorded as pseudo-2D spectra using low flip angle (5°) read pulses for excitation. A ^{13}C NMR spectrum of 16384 complex data points was recorded every 0.5 s. Data acquisition was started prior to substrate injection to minimize the experimental dead-time upon substrate feeding. All spectra were processed with extensive zero filling and an exponential line broadening of 10 Hz and integrated in Topspin 3.5 (Bruker). Integrals for time points above 15 seconds in Figure 3A are sum spectra of 4-20 transients to provide sufficient signal to noise upon fading hyperpolarization. Metabolites were identified by comparison to chemical shift assignments of standard compounds at pH 6.7 as summarized Table S1.

Hyperpolarized $[2-^{13}\text{C}]$ -fructose and $[6-^{13}\text{C}, 6,6'-^2\text{H}_2]$ -glucose substrate were used for in vivo NMR experiments, as the possibility of an H/D kinetics isotope effect slowing the reaction had rightly been raised in the literature.²⁶ Glyceronephosphate formed from $[2-^{13}\text{C}]$ -fructose and $[6-^{13}\text{C}, 6,6'-^2\text{H}_2]$ -glucose is not protonated at the position that is oxidized in the glyceraldehydephosphate dehydrogenase reaction. Nevertheless, glyceronephosphate accumulated as a glycolytic intermediate in these cases (Figure S1). Hence, GAPDH was a limiting step under physiological conditions and other factors than C-H cleavage limit reaction glyceronephosphate conversion. The finding was consistent with the observation of minor H/D isotope effects described in the literature when using C1 protonated and deuterated glyceraldehyde 3-phosphate and purified GAPDH.²⁷

NAD⁺/NADH perturbation

Experiments with experimental perturbation of the cellular NAD⁺/NADH ratio were performed as described above with the exception that acetaldehyde at the desired concentration was dissolved in 0.5 mL Milli-Q water and placed in the bottom of the receiving container for the hyperpolarized hexose in order to achieve a co-injection of acetaldehyde¹⁹ with the hyperpolarized glucose substrate. In order to evaluate the effect of a reducing environment on cellular glycolysis, yeast cells were maintained for 90 minutes in YPE medium (yeast extract (1% w/v), peptone (2% w/v), ethanol (2 % v/v)) prior to harvest, and an in vivo NMR experiment conducted as described above. Reference and perturbation experiments were acquired in triplicate on different cell cultures and using new DNP NMR preparations for in vivo NMR (Figure S3). Significant differences of pathway bottlenecks between the three groups were assessed using a Student's t-test (Figure 2B).

In silico modeling

The glycolysis model by Smallbone⁷ was used for the in silico modeling of *S. cerevisiae* glycolysis, and all simulations were carried out in the Mass-Action Stoichiometric Simulation (MASS) toolbox²⁸ v1.1.9, in Mathematica. The model uses enzyme kinetic parameters determined under standardized conditions, models isozymes, treats energy charge (adenosine phosphates) as variables and tolerates glucose concentrations below 0.5 mM (prerequisites to simulate effects of starvation). Experiments were simulated in silico as follows: the cell starvation was simulated by setting the extracellular glucose concentration to 0.1 mM for 20 minutes. In order to simulate the glucose pulse, the extracellular concentration of glucose was raised to 4 mM after the 20 minutes starvation period and the evolution of key metabolites and fluxes in yeast glycolysis were simulated for 60 seconds, i.e. the approximate time of the in vivo hyperpolarized NMR experiment. In order to simulate the co-injection of glucose and acetaldehyde, the extracellular concentration of glucose was raised to 4 mM and the internal concentration of acetaldehyde was raised to either 2.3 mM or 12

mM after the 20 min starvation period. Effects of energy charge and acetaldehyde concentrations were simulated by setting the initial glucose concentration to 4 mM, acetaldehyde concentration to 1.64 mM, ADP to 3.01 mM, and ATP to 0.75 mM such that the energy charge ratio would be 0.37, a value more realistic for starved cells (Figure S2).

RESULTS AND DISCUSSION

Effect of redox perturbation on glycolytic bottlenecks

Metabolic control in glycolysis occurs on a time scale of seconds⁴ and has been correlated to variations in the NAD⁺/NADH ratio (Scheme 2).^{30, 31} For instance, glyceronephosphate accumulation follows NADH accumulation in metabolically oscillating *S. cerevisiae*. Hence, a control of glyceronephosphate levels through NADH was deemed possible.³² Considering such correlations of glycolytic dynamics and NAD⁺/NADH ratio, we hypothesized that signal evolution for glycolytic intermediates in hyperpolarized ^{13}C NMR of glycolysis in living cells could be indicative of the cellular redox state.

Hyperpolarized NMR spectroscopy provides the possibility to noninvasively track the conversion of carbohydrate spins to glycolytic intermediates and end products.^{5, 12-18} In order to test the role of the cellular redox state in the regulation of glycolysis, established experimental perturbations were applied. In order to increase the cellular NAD⁺/NADH ratio, acetaldehyde was co-injected with hyperpolarized glucose substrate.^{19, 20} Acetaldehyde is easily and rapidly membrane permeable and serves as an electron acceptor that becomes reduced to ethanol by the reversible alcohol dehydrogenase (ADH) catalyzed reaction and in this manner regenerates NAD⁺ for the GAPDH reaction (Scheme 2). Accordingly, fluorescence measurements had shown an increase of the free cytosolic NAD⁺/NADH ratio when co-injecting acetaldehyde with a glucose bolus to *S. cerevisiae* cells (2.3 mM of acetaldehyde

Scheme 2. Schematic overview of Embden-Meyerhoff-Parnass glycolysis. Metabolic regulation occurs on the seconds timescale and changes to redox (NAD⁺/NADH) and energy (ATP/ADP) currencies occur on the seconds time scale.

and glucose), while a drop in the NAD^+/NADH ratio occurs when injecting glucose without acetaldehyde.^{4, 19} Here, we compared the influx of glucose carbon into glycolytic intermediates in the absence and in the presence of acetaldehyde. The experiments showed an impact of acetaldehyde on glycolytic dynamics. The accumulation of metabolites upstream of pathway bottlenecks, especially glyceronephosphate and 3-phosphoglycerate, was altered when increasing the cytosolic NAD^+/NADH ratio (Figure 1). Resultant spectra were consistent with the notion that glyceronephosphate formation follows NADH and thus is reduced at a higher NAD^+/NADH ratio.²⁴ Shortage of NADH under these conditions was witnessed by the fact that acetaldehyde reduction to ethanol slowed down, so that acetaldehyde formed from glucose started to accumulate (Figure 1, bottom).

Figure 1. Real-time spectroscopy of the reaction of glucose to intracellular metabolites in response to disturbed redox state. Co-injection of acetaldehyde as an electron acceptor increased NAD^+/NADH and reduced glyceronephosphate accumulation, while maintaining the conversion of 3-phosphoglycerate as a bottleneck. Abbreviations: 3PG, 3-phosphoglycerate; Acal, acetaldehyde; Pyr, pyruvate; GrnP, glyceronephosphate.

Figure 2. Effects of external electron acceptor (acetaldehyde) and electron donor (growth in ethanol) on rate-limiting steps in the glycolytic cascade. (A) Sum spectra of time-resolved rapid-injection experiments showed shifts of cascade bottlenecks. (B) The resulting areas under the curves for signals of upper and lower glycolysis depend on redox state. Ratios of glyceronephosphate and 3-phosphoglycerate signals under oxidizing, reducing and reference conditions, as well as variance of determinations in triplicate (see also Figure S3) are shown. Statistically significant

differences between the groups ($p < 0.002$, indicated by an asterisk).

The opposite effect was observed after incubation of *S. cerevisiae* cells in medium with 2% ethanol. Ethanol stress supposedly leads to NAD^+ shortage and hence reduced flux through GAPDH.³¹ Here, the presence of the reducing substrate lead to a higher accumulation of glyceronephosphate in phase with increased levels of NADH (Figure 2A). Hence, the abundance of glycolytic intermediates indeed seemed to reflect the cellular redox state and indicated that the NAD^+/NADH redox couple contributes to metabolic self-regulation under physiological conditions: the NAD^+ dependent enzyme GAPDH regulates glycolytic progression at the connection of upper and lower glycolysis in response to cellular redox state.

Conditions leading to increased NAD^+/NADH ratios were accompanied by lower accumulation of glyceronephosphate and higher accumulation of 3-phosphoglycerate than in the reference. In contrast, presence of 2% ethanol leading to lower NAD^+/NADH ratios³³ were accompanied by higher accumulation of glyceronephosphate and lower accumulation of 3-phosphoglycerate than in the reference (Figure 2A). This finding is consistent with ex vivo observations indicating a reduction of phosphoglycerate levels and an increase of metabolites upstream of GAPDH upon perturbation of the NAD^+/NADH ratio by ethanol.²¹ As a consequence, the areas under the curve for glyceronephosphate to 3-phosphoglycerate may be a suitable index for ratiometric assays of cellular redox state using rapid injection in vivo NMR spectroscopy. This ratio exhibits a nearly 10-fold change between oxidizing and reducing conditions (Figure 2B), which indicates a drastically faster conversion by GAPDH under oxidizing conditions relative to reducing conditions.³⁴ Hyperpolarized NMR thus emerges as a means of probing the cytosolic milieu, as signal positions of glycolytic intermediates had been shown to probe cytosolic pH,^{35, 36} while their dynamics report on cytosolic redox state as shown herein. Previously, hyperpolarized glucose had been used as a redox probe to observe the pyruvate/lactate redox couple in cells with

Scheme 3. Schematic overview of the effect on glycolytic dynamics after perturbation with (A) acetaldehyde and (B) ethanol.

high lactate fermentation.³⁷ This approach is not applicable to *S. cerevisiae* due to the absence of lactate dehydrogenase.²⁰

The impact of cellular redox state on glycolytic dynamics indicate that GAPDH operates close to equilibrium with an increasing driving force for flux through the step at higher NAD^+/NADH ratio (Scheme 3). Thus, a change in intracellular redox state results in a change of the upstream and downstream products according to mass action ratio.²⁰ These findings agree with recent suggestions that the control of glycolytic flux is distributed through several steps,⁷ GAPDH being one of them.³⁸

Comparison of in-cell NMR data and computational simulations

Progress in the understanding of cellular reactions has often been sought by the combination of experimental data with computational modeling, as metabolism is exceedingly complex.^{2,4} Although both hyperpolarized NMR and kinetic models of glycolysis describe glycolytic dynamics in molecular detail, to the best of our knowledge, hyperpolarized NMR data has not previously been used to test computational models in systems biology. For complex reactions, an enzyme kinetic formula can have on the order of 10 parameters³⁹ and the complete characterization of metabolic dynamics may remain beyond experimental reach. Hence, hyperpolarized in vivo NMR data may be used as a complement to either further characterize kinetic parameters when building kinetic models of metabolism or to evaluate the prediction accuracy of existing models.

Challenges in the interpretation of hyperpolarized NMR data arise from the fact that different kinetic aspects are convoluted in the observed signal, which is a consequence of employing non-equilibrium spin polarization. This non-equilibrium spin polarization approaches its equilibrium through spin-lattice relaxation (with time constant T_1) and is lost (and not recovered) by “read pulses”. These two experimental effects contribute to the dynamics of the observed signal, in addition to chemical reaction kinetics.⁴⁰ Signals of glycolytic intermediates observed in living cells through hyperpolarized NMR therefore cannot per default be easily interpreted in terms of absolute concentrations. The T_1 time is characteristic for the molecular site and dependent on the viscosity of the medium. Recent determinations of T_1 in the cytosol have indicated T_1 times on the order of 8.1-12.7 seconds for non-protonated carbon sites in small molecule metabolites.⁴¹ These values are comparable to the T_1 times of $[\text{U-}^{13}\text{C}, \text{U-}^2\text{H}]$ -glucose substrate carbons (approximately 10 seconds). As also the excitation of substrate and product signals is comparable, intracellular metabolite and extracellular substrate signal fade at similar rates. The integrals of hyperpolarized metabolite signals relative to the substrate were therefore used to approximate the evolution of metabolite signals (Figure 3A). This normalization is valid as less than 5% of the hyperpolarized glucose molecules are metabolized during the observation of metabolism (Figure S4), so that the substrate signal can be used as a relative quantification standard for metabolite signals. A quasi steady state is established within few seconds. Predicted errors due to the differential relaxation of metabolites during the few seconds between glucose uptake and metabolite detection are shown in Figure 3 as a shaded envelope.

Figure 3. (A) Signal areas of major glycolytic intermediates normalized to the substrate signal and compared to predicted steady state concentrations in a recent metabolic model.⁷ (B) Schematic depiction of signal influx into glycolytic intermediate pools pre-steady state that changes to quasi steady state within seconds.

This approximation of experimental intracellular metabolite dynamics is compared in Figure 3 with steady state concentrations observed from a recent⁷ kinetic model without the use of DNP NMR data input. Such models comprise network topology and enzyme kinetic parameters. The refinement of the models has hinged on increasingly refined determinations of enzymatic kinetic parameters under standardized in vivo-like conditions.^{6,7,29,42} Early models had described problems in modeling the kinetically complex GAPDH reaction.⁸ More recent models have evolved to a level that provided a convincing agreement in the comparison with the independent experimental data obtained by hyperpolarized NMR spectroscopy (Figure 3). Experimental in vivo data could thus become useful in improving the parametrization of computational models. Encouraged by the agreement of experimentally observed and computationally predicted influx of glucose into glycolytic intermediates, we undertook to experimentally study transient mechanistic responses in the upstart of glycolysis, and computationally simulated the experiment for comparison.

Changing Reaction Control in Living Cells on the seconds timescale

Previous experimental studies of cellular redox state upon a glucose pulse had shown a decrease of the NAD^+/NADH ratio within few seconds (Figure S5).^{4,19} As hyperpolarized NMR tracks glycolytic dynamics with sub-second time resolution and as signatures of glycolytic bottlenecks in *S. cerevisiae* change in the NAD^+/NADH perturbation experiments of Figures 1 and 2, we expected to observe a time resolved effect of changing NAD^+/NADH in the upstart of glycolysis. To probe pre-steady state reactions in the upstart of glycolysis, cells were starved for 20 minutes, because the NAD^+/NADH ratio increases upon carbon restriction²³ to yield a higher baseline for the subsequent decline of NAD^+/NADH . In addition, major glycolytic intermediates are expected to drastically decline under conditions of starvation.⁴³ Lower baseline levels of metabolites simplify the interpretation of hyperpolarized glucose signal influx into cellular metabolites, as undetected metabolites can contribute to the chemical kinetics of hyperpolarized metabolites.⁴⁰

Once a glucose bolus was applied to starved cell suspensions, 3-phosphoglycerate was more rapidly labelled than upstream glyceraldehyde-3-phosphate and fructose 1,6-bisphosphate

(Figure 4A). In addition, minor formation of phosphoenolpyruvate accompanied the initial formation of 3-phosphoglycerate (Figure S6). As 3-phosphoglycerate and phosphoenolpyruvate are formed downstream of the GAPDH reaction, the GAPDH reaction was not limiting in starved cells, but only became limiting during the upstart of glycolysis. Glycolytic bottlenecks thus shift from

Figure 4. (A) Formation of cellular fructose 1,6-bisphosphate (Fbp), glyceronephosphate (GrnP) and 3-phosphoglycerate (3PG) from exogenous hyperpolarized [^{13}C , ^2H]glucose. Labelling of lower glycolytic intermediates occurred at higher initial rates than labelling of upper intermediates. Signals fade as non-equilibrium hyperpolarization fades. Signal maxima are indicated by asterisks as guides to the eye. (B) Schematic representation of mechanistic changes detected in (A).

signatures detected under more oxidizing conditions to signatures detected under more reducing conditions (Figures 1 and 2). In vivo data thus indicated that a rate-determining step in the glycolytic reaction cascade dynamically changed in response to an external glucose stimulus within few seconds (Figure 4B). Increasingly upstream bottlenecks have conceivable functions in avoiding the exhaustion of cofactors used in upper glycolysis and thus contribute to metabolic self-regulation of glycolysis.⁸ Replicates of the experiments yielded signal shown in Figure 5A. Experimental data of glycolytic dynamics were compared to unbiased predictions from a computational simulation of the experiment using a recent kinetic model of *S. cerevisiae* glycolysis (Figure 5B).⁷ Simulations were conducted using this model in the MASS toolbox in Mathematica.²⁸ The computational simulation did not involve any data fitting or other changes in parameters describing the model, nor any use of experimental data as an input. Nevertheless, the model correctly predicted an earlier onset and higher initial rate of 3-phosphoglycerate formation as compared to bottlenecks upstream of GAPDH. The relative rates for experimentally observed and predicted influx of glucose into 3-phosphoglycerate, glyceronephosphate and fructose 1,6-bisphosphate pools are compared using opaque lines (of identical slopes in Figures

5A and B) as a guide to the eye. These relative initial rates were obtained by a linear fit of the initial experimental buildup of 3-phosphoglycerate, glyceronephosphate and fructose 1,6-bisphosphate signals. The initial rate of glucose influx into 3-phosphoglycerate was 1.4-fold higher than the initial rate of influx into glyceronephosphate and 2.5-fold higher than the initial rate of influx into fructose-1,6 bisphosphate.

Figure 5. (A) Integrals of cellular fructose 1,6-bisphosphate (Fbp), glyceronephosphate (GrnP) and 3-phosphoglycerate from exogenous glucose, including two reproductions. The traces of the different signal areas were stacked for clarity. (B) Computationally simulated response of the upstart of glycolysis in starved *S. cerevisiae* cells. The simulation qualitatively reproduced the trend shown in (A) and did not involve any fitting to the experimental data. Trend lines for the influx of glucose into the metabolite pools were added as guides to the eye, where identical slopes were used in (A) and (B).

A comparison of experimental data and simulation yields additional insight into the current mechanistic understanding of glycolysis. Taking into account a lower energy charge in the starved cells (~ 0.4)^{2, 44, 45} as compared to the standard parametrization (>0.8)⁷ improved the agreement of absolute time scales for label influx into glycolytic intermediates in the simulation (Figure S2). The demand for ATP in starved cells may rationalize such acceleration of glycolytic flux.⁴⁶ Simulations correctly predicted rapid accumulation of 3-phosphoglycerate, glyceronephosphate, fructose 1,6-bisphosphate upon a glucose pulse, and significantly lower accumulation of fructose 1-phosphate and glyceraldehyde-3-phosphate. Overall, these comparisons indicate that metabolic models have improved over time to paint a rather accurate picture of glycolytic dynamics. Thus, metabolic models can be useful in simulating physiological changes (such as redox and energy state) that rationalize experimentally observed glucose label influx into glycolytic intermediates.

CONCLUSION

In conclusion, mechanistic aspects of cellular responses to the availability of glucose were probed by the combined use of experimental models affecting cellular redox state (acetalde-

hyde/ethanol or starvation),^{19, 23, 31} rapid injection NMR spectroscopy and computational simulation. Experimental results indicate that rate-limiting steps in glycolysis change within seconds during the upstart of glycolysis in parallel to rapidly changing redox state. Such self-regulation can sustain glycolysis at low substrate concentrations, but avoids an exhaustion of cofactors in upper glycolysis when ample substrate is available.⁸ The findings support long-standing suggestions that NADH levels control metabolite levels in upper glycolysis. Acetaldehyde had been known to elicit dynamic responses in vivo, most notably by synchronizing glycolytic oscillations via its effect on the NAD⁺/NADH ratio and the GAPDH reaction. Here, we observed that acetaldehyde shifts glycolytic dynamics by maintaining a state that is reminiscent of glycolytic upstart. Experimental findings were supported by in silico simulations using the currently best working models of *S. cerevisiae* glycolysis. Kinetic models of central metabolism appear to have achieved a level of quality that permits correct mechanistic predictions of unanticipated biochemical events. The combination of scarcely available kinetic observations in living cells and computational modeling thus provides a promising method for characterizing metabolic control with increasing mechanistic detail.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.

Figures of supporting experimental and simulation data. Experiments showing initial formation of PEP in *S. cerevisiae* upon a pulse of hyperpolarized glucose. Experimental reproducibility and assignments. Schematic depiction of *S. cerevisiae* cytosolic free NAD⁺/NADH ratios expected upon perturbation with acetaldehyde and predicted from simulation. Predicted trends for the effects of acetaldehyde co-injection during startup of glycolysis. The Mathematica notebook and model are provided alongside PDF and CDF versions of the notebook.

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Notes

The authors declare no competing financial interest.

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