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The multifarious and dynamic regulation of the living cell

van Eunen, K.

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Summary

One of the key characteristics of a living organism is its ability to adapt to sudden changes in the environment. The response of organisms to these changes is regulated at various levels of cellular organization, including the processes belonging to gene expression and metabolism. This thesis focuses on the interplay between the gene-expression cascade and metabolic pathways when the organism adjusts its physiology to new environmental conditions.

Regulation Analysis is a novel methodology to quantify and dissect the regulation of metabolic fluxes into gene-expression and metabolic components. Preceding the work described in this thesis, Regulation Analysis had been applied to describe the transition from one steady state to another. With the latter approach one will miss, however, the primary regulatory events during the adaptation of an organism. Therefore, a time-dependent extension of Regulation Analysis was applied in this thesis, so as to obtain more insight into the primary adaptation strategies of the organism.

The baker's yeast *Saccharomyces cerevisiae* is an important model organism for the regulation of cellular processes in eukaryotes. Besides, it has many classical and modern applications in industry. The research in this thesis was part of a larger project, called *Vertical genomics: from gene expression to function, ...and back*. In the project *S. cerevisiae* was cultivated under highly standardized conditions and then faced with various challenges. Apart from nitrogen starvation, which has been the focus of this thesis, these included elevated temperatures, anaerobic or aerobic glucose excess and various degrees of hypoxia. Under all these conditions the integrated response of energy metabolism (notably glycolysis) was quantitatively dissected into contributions by gene-expression and metabolic regulation. Altogether, the project has yielded a broad view on the mechanisms underlying the regulation of cellular fluxes.

Nitrogen starvation has a strong impact on cellular physiology. In the absence of nitrogen in the environment, cells synthesize new proteins by degrading existing proteins. The glycolytic and fermentative capacity of yeast cells, *i.e.* the rate at which it can convert glucose to ethanol and CO₂ under anaerobic, glucose-excess conditions, tends to decrease during prolonged (24 h) starvation for nitrogen. Previous studies have shown that during nitrogen starvation the flux through glycolytic enzymes is regulated by metabolic regulators as well as via adaptation of gene expression. The quantitative contributions of each of these differed strongly between enzymes and conditions. This was unexpected, since the prevailing view was that unspecific bulk degradation of proteins via autophagy would be dominant. If the latter were true, one should have expected a proportional decrease of the catalytic capacities

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of all glycolytic enzymes, which would have translated into a pure gene-expression regulation. In Chapter 2 and 3, the regulation of the glycolytic flux upon nitrogen starvation was therefore monitored in time during 24 hours. Yeast cells were grown under aerobic glucose-limited chemostat conditions in excess of nitrogen. When steady state had been reached, the cultures were shifted to nitrogen-starvation conditions, in the presence of an excess of glucose to prevent dual starvation. Simultaneously, the cultures were brought to batch conditions, since the cells would not grow when starved for nitrogen. The fluxes through glycolysis and its branches were monitored, as well as the V_{max} 's of the glycolytic and fermentative enzymes. The latter were used as a quantitative readout of gene-expression regulation. From these data it was calculated to what extent the changes in the fluxes were regulated by gene expression and/or metabolism, according to the theory of time-dependent Regulation Analysis. The main conclusion of Chapter 2 was that the regulation of the glycolytic flux upon nitrogen starvation is distributed among gene expression and metabolism in an enzyme-specific manner, even in the first hours after removal of nitrogen. This demonstrates that even the initial response is highly specific, rather than dominated by unspecific bulk degradation of protein. In Chapter 3 it was subsequently shown that the growth conditions prior to the starvation played an important role in the regulatory strategy of the yeast. In cells grown under respirofermentative conditions gene-expression regulation was dominant, while in cells from respiratory cultures metabolic regulation dominated. Under both growth conditions, however, metabolism played some role in the regulation of the flux. Therefore, the changes in glycolytic metabolites and allosteric regulators were analyzed. The results could explain some of the observed metabolic regulation, but not all.

In order to further investigate the nature of the metabolic regulation during nitrogen starvation, I planned to implement the obtained data into an existing kinetic model of yeast glycolysis. However, before this could be done, the V_{max} values obtained in Chapters 2 and 3 were redetermined under conditions that resembled the intracellular environment. So far, they had been measured under assay conditions that were optimized for each enzyme separately to achieve a high activity. The latter assay conditions were not necessarily similar to the *in vivo* environment. Therefore, in Chapter 4 an *in vivo*-like assay medium was developed to measure enzyme activities (V_{max}) under more physiological conditions. Intracellular concentrations of the small ions present in the yeast cytosol were estimated under standard growth conditions. A single assay medium was composed that reflected these *in vivo* conditions. We propose that the assay medium presented in Chapter 4 will be a new standard for enzyme-activity assays in yeast Systems Biology.

In Chapter 5 the V_{max} 's of the glycolytic enzymes were measured in the new *in vivo*-like assay medium, in starved and non-starved cells originating from respiratory and respirofermentative cultures. When the new dataset was implemented in an existing

kinetic model of yeast glycolysis, the model did not reach steady state, while our experimental system did. Therefore, three additional modifications were made to the model: (i) addition of the known inhibition of hexokinase by trehalose 6-phosphate, (ii) redetermination of the kinetic parameters of glyceraldehyde-3-phosphate dehydrogenase under *in vivo*-like conditions and (iii) addition of the known activation of pyruvate kinase by fructose 1,6-bisphosphate. These adaptations improved the model such that steady states were obtained for all experimental conditions studied. However, for only one condition the predicted fluxes and metabolite concentrations corresponded well with the measured values. For the other three conditions extremely high fructose 1,6-bisphosphate concentrations were predicted. In order to bring the concentration of fructose 1,6-bisphosphate down in the model, whilst maintaining a good correspondence to the measured flux, minor adaptation to the kinetics of hexokinase and glyceraldehyde-3-phosphate dehydrogenase were necessary. This resulted in a good fit for another two conditions, while the correspondence between the model and the experiment for the fourth and last condition under study improved substantially.

During prolonged nitrogen starvation, protein degradation is essential for maintaining sufficiently high free amino-acid levels in the cell. We therefore hypothesize that the decrease of the V_{max} 's of glycolytic enzymes observed in Chapter 2, 3 and 5 should be attributed to degradation of glycolytic enzymes during nitrogen starvation. In Chapter 6 we have developed a method for measuring protein degradation rates in standardized yeast cultures. The method was based on an existing SILAC (stable-isotope labeling by amino acids in cell culture) technique in which the loss of stable-isotope label from proteins was monitored in time. The key differences between our method and the existing one are (i) our use of a strain which is prototrophic for all amino acids and (ii) the use of ^{15}N -labeled ammonium sulfate instead of labeled amino acids in our culture medium. These changes of the cultivation protocol had dramatic consequences for the label fluxes. The experimental results and the simulations of a computational model showed that label from degraded proteins was recycled into newly synthesized proteins, and that this influenced the estimated protein-degradation rates substantially. Based on the computational model we propose that additional determination of label incorporation in the amino-acid pool will allow to resolve the protein turnover rates in cultures growing on ammonium instead of amino acids. The method developed in this chapter is, however, not directly suitable for measuring protein degradation in nitrogen-starved cells, since ^{15}N -labeled ammonium sulfate is used. A solution could be to use ^{13}C -labeled glucose instead of labeled nitrogen.

This thesis showed quantitatively how different levels of cellular regulation play a role in the adaptive response of yeast cells to changes in the environment, and specifically to nitrogen starvation. In the General Discussion to this thesis (Chapter

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7) I analyze, based on results from the Vertical Genomics project as well as literature data, whether a general strategy of flux regulation in yeast can be identified. This turns out not to be the case. It appears however that cells that remain under respirofermentative conditions during a physiological challenge tend to invoke more gene-expression regulation, while a shift between respirofermentative and respiratory conditions invokes an important contribution of metabolic regulation. This analysis may be a first step towards discerning strategies of flux regulation. The observed complexity of regulation now brings up the question what is the advantage of this highly distributed and condition-dependent flux regulation.