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The effect of altered expression of transcriptional regulators of catabolism on the transcription profile and physiology of Saccharomyces cerevisiae

Schuurmans, J.M.

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Chapter 6

General Discussion

Retrospect

In this thesis several aspects of central carbon catabolism have been addressed in relation to changes in expression of various catabolic regulators, with a focus on fermentative and respiratory capacity. Although this research started with characterizing strains with altered expression of *GCR* (Glycolytic Regulator) genes (*GCR1* and *GCR2)*, it turned out that strains harboring deletions of these genes were not easily obtained, and were even harder to work with. However work on *GCR* deletion strains has been continued by other researchers. Particularly the work of H. Uemura and co-workers has shown the function, by analysis of deletion mutants of *GCR1* and *GCR2,* in maintaining proper glycolytic activity and their effects on other metabolic pathways (Sasaki and Uemura, 2005). *GCR1* also functions in activating transcription of ribosomal genes and supporting maximum expression of G1 cyclins (Barbara, et al., 2007a) and is part of the yeast "coilome", a protein complex which is involved in transcriptional regulation (Barbara, et al., 2007b). Additionally, unequivocal and often irreproducible results have been obtained with strains modified in *GCR1* gene expression, (J.T. Pronk, personal communication).
For these reasons, we decided to focus our studies on the characterization of strains with increased respiratory capacity under glucose excess and aerobic conditions. During the research sometimes different media or strain backgrounds were used, so care should be taken when directly comparing results. A first transcriptional characterization of three such strains in batch culture is described in chapter 2. In this chapter some earlier findings on the *hxk2Δ* strain and the *HAP4* ↑ strain are confirmed and several new aspects are identified. Thus it is shown that there are synergistic effects when modified expression of both regulators is introduced in one and the same strain: an *hxk2* deletion strain with overexpression of *HAP4* shows an even larger increase in respiration than both single mutations separately. This experiment illustrates that additional activator protein (*HAP4*) can enhance the respiratory chain activity even further. Indeed, biomass yield of this mutant was increased with respect to the wild-type, to such an extent that the *hxk2ΔHAP4* ↑ strain nearly reached the theoretical maximal biomass yield for baker's yeast. In addition to confirming the findings of increased respiration and lack of glucose repression for the *hxk2Δ* strains (Diderich, et al., 2001a; Hohmann, et al., 1999), several additional results were obtained, i.e. a change in the transcription of the genes needed for zinc and iron homeostasis.
The *hxk2*Δ and *HAP4* ↑ strains were then investigated for their performance under anaerobic conditions, as described in Chapter 3. With respect to fermentative capacity it was found that the *HAP4* ↑ strain does not significantly differ from the wild-type. This again illustrates that the Hap-system functions at the physiological level of respiration, or possibly at that of the tri-carboxylic acid cycle, but not at that of glycolysis. In contrast, our studies on the fermentative capacity of the *hxk2Δ* strain support the idea that this regulatory system is involved in the upper part of glycolysis and even at the level of glucose sensing and carbon source uptake: deletion of *HXK2* results in a large decrease in fermentative performance on glucose, but an increase in performance on maltose. The cause of this difference was investigated at the level of transcription profiles, activity of glycolytic enzymes and rate of glucose/maltose transport.

To investigate the transcriptional changes in the *HAP4* ↑ strain in more detail, an extensive analysis of transcript profiles of the *HAP4* ↑ strain grown under nutrient-limited as well as unlimited growth conditions was carried out and is presented in Chapter 4. Clearly, the effects of Hap4p on the biochemistry of the cell turn out to be complex but as a common feature it is concluded that Hap4p regulation is related to oxygen metabolism.
Chapter 5 describes a study we have carried out in collaboration with our colleagues from the University of Stellenbosch on the functional interpretation of the Michaelis-Menten affinity constant (K_m) of the glucose transporters and the overall affinity of the organism for growth on glucose (K_s) in terms of the growth rate control of the glucose transport machinery of the cell.

In conclusion, this thesis describes several new findings on the physiology of yeast strains with an increased respiratory catabolism; nevertheless many questions in this field are still unanswered and new questions have emerged.

Hexokinase II signaling

Even though the phenotype of the *hx2kΔ* strain was identified back in 1977 (Zimmermann and Scheel, 1977), the details of the changes this mutation causes, and how these changes are brought about, is still not fully understood. It was reported that approximately 14% of Hxk2p is localized in the nucleus in glucose-grown cells (Herrero, et al., 1998), and that it is there were Hxk2p fulfills its regulatory function. Although many now believe that the catalytic activity of Hxk2p in glucose phosphorylation does not participate in transmitting the signal for glucose repression, a strain with a chimaeric hexose transporter protein (Otterstedt, et al., 2004) shows in fact a similar phenotype as a *hxk2Δ* strain, indicating that at least a part of the signal is created there because the glucose sensing Snf3p-Rgt2p pathways is still present in both strains. The chimaeric hexose transporter strain (Otterstedt, et al., 2004), is a transporting system made up from parts of the low affinity hexose transporter Hxt1p and parts of the high affinity hexose transporter Hxt7p and the constructed strain expresses only this single chimaeric protein as a hexose transporter. Presumably, the Hxt7p part is located partly in the cytosol, which could possibly signal a low-glucose condition. Although this could be mediated by Mth1p (which can bind to the C-terminal tail of the glucose sensors and is part of the Snf3p-Rgt2p signaling pathway), mutants in the Snf3-Rgt2 pathway do not show such a large shift towards respiration-supported growth, suggesting that Hxk2p is indeed involved in transferring a signal from the surface of the cell to the nucleus. Additionally, it has been shown that intracellular glucose can reduce transport rates up to 50% (Teusink, et al., 1998), which would imply that it is imperative for a cell to keep intracellular glucose levels as low as possible, which requires glucose-phosporylation. These observations could still support a mechanism of action of Hxk2p near the cell surface. On the mode of action of Hxk2p in the nucleus much more progress has been made. Several interactors of Hxk2p have been identified, namely Med8p (de la Cera, et al., 2002) and the transcriptional repressor Mig1p (Ahuatzi, et al., 2007). These results, together with the action of the Snf1p kinase in response to activation of the PKA

pathway, show that glucose repression converges at the Mig1p repressor, as both Snf1p and Hxk2p regulate the phosporylation level of Mig1p. However, it then still remains unanswered as to why only *hxk2* deletion results in a nearly completely de-repressed cell, and not the *mig1Δmig2Δ* strain. Therefore, it would be interesting to resolve whether the *hxk2Δ* phenotype can be re-created by the combined mutation of several downstream factors, as deletion of only the *MIG* proteins is not enough.

Changes in transcription of genes, required for iron and zinc homeostasis

Specifically for the *HAP4* ↑ strains, and to a lesser extent in the *hxk2Δ* strain, large changes in the expression of genes for zinc and iron homeostasis were found (chapter 4). For iron homeostasis, exemplified by the Rcs1p element, an increase in genes involved in iron uptake and utilization was found under carbon-limited growth conditions. Under these conditions the respiration rate of both strains is the same *in vivo*, but it should be realized that the actual the maximal respiratory capacity of the *HAP4* ↑ strain could very well be higher than that of the wild-type. As such, the cells would have an increased need for haeme. If a cell is poised for increased respiratory capacity in some way, in the case studied here by changes in the regulatory network, upregulation of the synthetic capacity of components of the respiratory chain should follow, in accordance with the observation of increased expression of genes controlled by Rcs1p.
We found that a *HAP4* ↑ strain also downregulates genes needed for transport of zinc, which suggests altered requirements in zinc homeostasis. At first, a relation with the zinc-dependent superoxide dismutase and a manganese-dependent superoxide dismutase was thought to be involved, by reasoning that the increased amounts of mitochondria would result in higher levels of Radical Oxygen Species (ROS). However, changes in expression of these genes were not observed. Alternatively, a link between the different *ADH* iso-enyzmes was proposed. During growth on glucose *ADH1* and *ADH3* are the most abundant alcohol dehydrogenases, which both require 4 zinc molecules, whereas *ADH4* only requires 2. Other reports showed that indeed when cells are zinc-deprived *ADH1* and *ADH3* are repressed and *ADH4* expression is increased to overcome the low availability of zinc (Bird, et al., 2006). And indeed large changes of the expression of *ADH4* were found under the conditions tested in Chapter 4, as well as changes in the expression of the *ZRT* (zinc transport) genes. Interestingly, the effects of zinc at the physiological and molecular level are still largely unknown and much work is currently performed to further elucidate various problems associated with zinc availability in for instance beer fermentations, where - if zinc ions become limiting - the fermentation process becomes sluggish. A recent publication on zinc availability showed that the response to a zinc limitation is very pleiotropic, including physiological changes related to oxygen availability, respiration and ethanol formation (De Nicola, et al., 2007). The role of zinc in mitochondrial biogenesis is far from elucidated; its functional role in respiration has been demonstrated (*e.g.* it is reported that cytochrome c oxidase is inhibited by zinc), but scarcely characterized. Therefore, we can at this point not draw any conclusions as to how Hap4p and zinc metabolism are related but we suggest that the

observed decrease in zinc regulatory genes in the *HAP4* ↑ mutant is connected to better functioning of the respiratory chain and the increased amount of functional mitochondria in this mutant.

Improving fermentative capacity

The ultimate goal of the research performed in this thesis was to improve our knowledge as to how the balance between fermentation and respiration is regulated, in order to improve the fermentative capacity of yeast cells under anaerobic conditions. Ideally, improved strains should retain their ability to grow fully respiratory when needed, e.g. during their industrial production process. Many approaches have been used to increase this fermentative capacity, for instance by overexpressing the enzymes involved in the lower part of glycolysis (Smits, et al., 2000). This resulted in an increase of the glycolytic flux under several specific conditions. However, generally it shifted rate control in this pathway from one enzyme to another. As the *HAP4* ↑ strain showed, changing the expression level of the transcriptional regulator which controls the pathway, leads to an increase of virtually *all* enzymes involved and as such increases the flux through the pathway as a whole. Increasing the glycolytic performance could perhaps also be achieved with the following approach: By changing key signals in the cell (*e.g.* PKA activity), via mutation of key proteins in these signaling pathways, a response could be achieved, which is independent of the environment in which the cells reside. As the *hxk2Δ* strain showed, omission of the signal transmitted by Hxk2p, completely changed cell metabolism and made these cells fairly unresponsive to varying glucose concentrations. To increase the fermentative capacity perhaps a similar removal or increase of signals might accomplish a high fermentative capacity. At high growth rates, industrial baker's yeast has a much higher fermentative capacity than at low growth rates (Van Hoek, et al., 1998). The trick should then be to "fool" a slowly growing cell into sensing that all the necessary signals are there to conclude that it grows fast. A first start would be to investigate further the role of the PKA pathway and its interaction with the Msn2-4p pathway, in response to nutrient starvation. Some effects of the Msn2-4p pathway were observed in nutrient-limited chemostat cultures. If one could decouple the PKA pathway from the Msn2-4p dependent starvation response (a way to start would be a *msn2-4Δ* mutant), and combine this with a hyperactive PKA mutation (*ira1Δ* and *ira2Δ*), a cell might be tricked into a high fermentative output, even at near carbon source depletion. Although this will most likely raise problems in parts of intermediary metabolism, for a high glycolytic flux this could be an option worthy to investigate.

Systems Biology – towards a virtual cell and its synthetic descendants

Baker's yeast has been a very important research object for many years now, for many reasons, such as improving its quality for the baker, improving growth characteristics as a cell factory, and as a model organism for better understanding of higher eukaryotes. Research in the eighties and nineties mostly focused on the isolation and characterization

of single genes of interest. However, nowadays many tools are available to perform large scale analyses, such as genome-wide transcription assays, highlighting changes in transcription of every gene available in yeast upon selected perturbation, whether these are nutrient pulses, stress conditions, or the effect of a gene deletion. In addition, results of more and more different types of these large scale analyses are becoming available, such as quantifying wide ranges of proteins (de Groot, et al., 2007; Kolkman, et al., 2006)or metabolites (van der Werf, et al., 2007; Wu, et al., 2005)of the cell under a certain condition. More and more tools are designed to dissect the various aspects of regulation on different levels. For instance, regulation analysis can be used to dissect the overall physiological changes in a pathway into those brought about by transcriptional events and those due to metabolic events such as kinetics, feed back loops, product inhibition etc. (for more information about regulation analysis see (Rossell, et al., 2005). One aspect of all these large scale assays is the enormous amount of data they lead to. Fortunately, now with more computer power, these data-sets can be more easily analyzed. Proper design of data handling, allowing for exchange between research groups, could lead to a valuable and manageable data set that may form the basis for modelling cellular aspects such as regulation, growth and physiological performance. Using this approach of integrating every data point available, allows for much greater understanding of the underlying mechanisms of how cells work, than by just characterizing every gene as a single unit, as it incorporates the effects of interactions and functioning of networks within the cell. This can be exemplified by our study (chapter 3) with respect to the cause as to why an *hxk2Δ* mutant performs so much worse than the wild-type under anaerobic conditions. Although many levels (transcriptome, enzyme activity, transport), have been studied, none showed a clue as to why an *hxk2Δ* mutant performs so poor. Integrating data on the metabolomics of wild type and mutant strains would be the proper approach to further understanding of the events going on inside the mutant cells.

The studies presented in this thesis show once again that it is not justified to consider the yeast cell as a *simple* model system. Its glycolysis is one of the longest and most intensively studied subjects in the life sciences and still has many secrets for us. This thesis illustrates the complexity of glycolytic regulation, as well as the intricate interaction between glycolysis and fermentation, respectively respiration. Only a concerted effort of many disciplines, from genetics through enzymology and physiology to data analysis and mathematical modelling can resolve the functioning and regulation of this central and essential catabolic module.