Chapter 5

Conclusions and outlook

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Summary of the thesis

In this thesis we investigated the role of fructose-1,6-bisphosphate (FBP) as a flux-signaling metabolite, and explored its participation in the regulation of the metabolism. In Chapter 2, we biochemically tested the putative interaction between Hxk2 and FBP, suggested by a novel mass spectrometry methodology[1], where Hxk2 showed conformational changes when in presence of FBP. We did not find any indication of a direct interaction with FBP, which led us to explore the possibility of secondary effectors, such as metal ions. Indeed, we found that zinc affects the stability and the activity of Hxk2, and that FBP, by acting as a chelator, restores it. In Chapter 3, we further explored the role of FBP in altering the conformation of seven other proteins that were suggested to interact with this compound. We found that the stability of these proteins was not influenced by FBP, and also no binding could be identified. FBP also did not affect the activity of two enzymes tested. All together, these results indicate that there is no direct interaction of FBP with the studied proteins. In Chapter 4, we investigated the role of FBP in the regulation of two transcription factors: CggR and Cra. We determined that the concentration range, in which FBP modulates the interaction of CggR and the DNA operator, lies in the millimolar range. For Cra, where an interaction with FBP and its regulatory role were still a point of debate in the literature, we provided experimental prove that FBP does not interact with Cra and does not modulate the activity of this transcription factor. Overall, we concluded that FBP does not interact directly with the proteins studied in Chapter 2 and 3, however, we hypothesized that FBP could exert an indirect regulatory role by chelating metal ions, which would globally modulate the activity of enzymes in a glycolytic flux-dependent manner. Furthermore, we concluded that FBP, by acting as a flux signaling metabolite, provides an essential link between flux signals and gene expression regulation by modulating the activity of CggR.

Future outlook

On the basis of my thesis work, I suggest a novel flux-dependent regulation of the metabolism, where FBP, by acting as a chelator, indirectly regulates the activity of enzymes. If confirmed, my hypothesis could shed light into a new element of the flux-sensing regulatory system, where the activity of metabolic proteins can be globally but indirectly regulated by metabolites, which chelate ions. Below, I further discuss this hypothesis, and what future research could be performed to continue this work and to improve the understanding of FBP as a chelator and its participating in the flux-dependent metabolic regulation.

Metal ions play crucial roles in cellular processes. An important determinant of their functional relevance is the fact that a wide variety of enzymes requires metals for their catalytic activity[2]. There are indications that the participation of metal ions started early in evolution, even before the existence of enzymes. Recently, a study on the reverse tricarboxylic acid (rTCA) cycle, an anabolic biochemical pathway, explored the proposed geochemistry origin of this pathway, which is expected to exist before the advent of enzymes, RNA or cells[3]. It was observed that most of the reactions from the rTCA were feasible in an acidic aqueous solution promoted by the metal ions Zn^{2+}, Cr^{3+} and Fe^{0}, without the use of enzymes, supporting the feasibility of primitive anabolism in an acidic, metal-rich reducing environment.

Due to the essentiality of metal ions, an optimal intracellular concentration of each particular metal ion is required for homeostasis[4]. Metal ions are likely to form complexes with dissociable ligands, affecting the intracellular concentration of available metal ions. Chelating molecules with specific metal binding sites and high affinity for metal ions can be used to modify the chemical, biological and the activity of associated proteins. Natural and synthetic chelators, as well as competing metal ions, can determine the relative activity of the enzyme reaction influencing an overall metabolic response[5,6]. According to my hypothesis, the flux-signaling metabolite FBP, by acting as a chelator, could regulate enzymatic activities indirectly via chelating metal ions in a flux-dependent manner. In Chapter 2, we have tested this hypothesis with Hxk2, where we observed that zinc decreased the stability and the activity of Hxk2, and both effects were restored by FBP. In Chapter 3, where no indication of direct interaction with FBP was observed, we showed that metal ions are required for activation and inhibition of all the studied proteins. However, a potential chelator effect of FBP still needs to be evaluated in these proteins.

Furthermore, in order to test our hypothesis further, studies to (i) identify other flux-signaling metabolites, and to (ii) identify among these flux-signaling metabolites the ones that exhibit chelating properties, are required. In fact, the metabolite citrate, an intermediate in the later stages of TCA cycle, has been recently identified as a potential flux-signaling metabolite (unpublished data), and has been previously shown to exhibit cation-chelating properties[7,8].

Another aspect that needs to be studied is the role of metal ions on the activity of enzymes, which can be tested as performed in Chapter 2, by determining the enzymatic

activity in presence of specific metal ions, and in combination with chelators. However, with this approach, only one protein and one metal ion can be tested at a time, resulting in extremely time consuming experiments. In fact, the method developed by Feng and co-workers could be used as a screening method to determine structural conformation of proteins in presence and absence of a certain metal ion, and after the addition of flux-signaling metabolites, which act as chelators. However, in order to accurately establish the biological function of the results by such methods, it is essential to validate these results using biochemical and biophysical methods.

Determining the role of different metal ions in the activity of metabolic enzymes is a crucial step, however, it is not sufficient for a comprehensive and systematic understanding of this novel flux-dependent metabolic regulation. Towards proofing the mechanism hypothesized in this thesis and its physiological relevance, the next step will be to investigate whether the free metal ion concentration in the cell changes under high and low concentrations of FBP. Methods such as the Donnan membrane technique[9], AGNES (absence of gradients and Nernstian equilibrium stripping)[10], and fluorescence-based methods that use small-molecule sensors or protein-based biosensors[11] have been used to measure free metal ion concentration, and could here be used.

Next, targeted perturbations would be needed to determine whether the altered free metal ion concentration indeed changes the activity of enzymes *in vivo*. Measuring *in vivo* activity of enzymes could be done in the way Link and co-authors[12] did. To finally proof the effect of the altered metal ion concentration, it would be necessary to replace the endogenous enzyme with an enzyme that would not respond to the metal ion changes, which however will represent a significant challenge.

Thus, to prove the existence of a flux-dependent mechanism, where FBP and other flux-signaling metabolites, by acting as chelators, can modulate enzymes indirectly, and consequently regulate metabolism, is extremely challenging. However, understanding new ways in which metabolism can be regulated will increase the knowledge of a comprehensive regulatory system where these regulatory elements are connected.

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