

## Role of Mitochondrial Carriers in Metabolic Engineering

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Metabolic engineering is generally defined as the targeted and purposeful alteration of cellular metabolic pathways to increase the cellular production of a certain substance [1]. Metabolic engineering of microorganisms has great potential for the low cost and environmentally friendly production of chemical feedstocks and novel compounds.

In eukaryotic cells a number of metabolic pathways are separated not only by means of different enzyme systems that catalyze their forth and back reactions (anabolism and catabolism), but also by multiple compartments separated from each other by membranes. The mitochondrial compartment plays a key role in energy and central metabolism of eukaryotic cells. The inner mitochondrial membrane is a selective barrier that controls energy producing pathways as well as carbohydrate, fat, and amino acid metabolism. Transport of metabolites (di- and tricarboxylates, amino acids, keto acids as well as nucleotides and coenzymes/cofactors) across the inner mitochondrial membrane is mostly carried out by a number of transporters that belong to the mitochondrial carrier family (MCF) [2,3]. The genome of the yeast *S. cerevisiae* encodes 35 proteins belonging to the MCF [4]. 34 of them are localized to the inner mitochondrial membrane except one, Ant1p, which is a peroxisomal adenine nucleotide transporter [5]. The transport specificity of 20 of these carriers has been unveiled [6,7]. The function of the remaining 15 transporters is still unknown [2].

Many metabolic engineering strategies rely on the manipulation of enzyme levels to achieve the amplification, disruption or addition of a metabolic pathway. Modification of expression level of specific intracellular transporters is an unexplored tool for both studying and engineering metabolism. It has been demonstrated that deletion or over expression of genes encoding mitochondrial carriers (MCs) can affect the subcellular concentration of their substrates causing a wide range of different phenotypes [6,8,9]. Consequently it is reasonable to hypothesize that also intracellular fluxes may result affected. Moreover, it is also possible that MCs might control the production rate of metabolites whose biosynthetic pathways are partially or completely located within mitochondria.

The importance of fluxes of substrates and products across mitochondrial membrane has been demonstrated or postulated for a number of biotechnological processes. The production of citric acid and itaconic acid are two good examples.

The fungus *Aspergillus niger* has an intrinsic ability to accumulate and secrete citric acid in specific culture conditions (i.e. sugar excess, acidity, nitrogen, phosphate, manganese and iron limitations) [10]. Although citric acid synthesis by *A. niger* has been described in details, the biochemical mechanism by which it reaches about 90% of final yield is still poorly understood. Citrate is synthesized by citrate synthase inside the mitochondrial matrix and, in specific conditions; it is exported out of mitochondria instead of being converted inside the organelle to cis-aconitate. The export of citric acid from the mitochondrial lumen into the cytosol is most probably catalyzed by a transporter belonging to the MCF (a tricarboxylate carrier) [11], whose function has been proposed to be essential for citric acid accumulation. Since this putative tricarboxylate carrier competes

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directly with aconitase for citrate, if its affinity for citrate were much higher than that of aconitase, then the carrier would export citrate out of the mitochondrial matrix. Alternatively the inhibition of the TCA cycle has been suggested as the biochemical mechanism explaining citrate accumulation, but no compelling evidence has been provided [10]. The tricarboxylate carriers of mammalian cells and yeasts export citrate from the mitochondria by counter transport with malate [12]. This could also happen in *A. niger* where malate is produced from oxaloacetate in the cytosol by malate dehydrogenase and is substantiated by the fact that malate accumulation has been shown to precede citrate secretion [10,13]. The *A. niger* citrate MC has not been identified. A BLASTP analysis reveals that *A. niger* genomes encodes at least three potential tricarboxylate carriers. The identification of these isoforms involved in citrate accumulation and their biochemical and physiological characterization are essential to understand and improve this biotechnological process.

Itaconic acid is an important building block in the chemical industry and a platform chemical for the synthesis of potential biofuels such as 3-methyltetrahydrofuran [14]. Similarly to other organic acids such as citric acid or lactic acid, itaconic acid is mainly supplied by a biotechnological process employing in this case the fungus *A. terreus* [15]. The biosynthesis of itaconic acid involves the shuttling of intermediates between cytosol and mitochondria. A key enzyme of the itaconic production pathway, cis-aconitate decarboxylase (CadA), is localized in the cytosol, whereas the preceding enzymes in the pathway, namely citrate synthase and aconitase, which synthesize cis-aconitate, are placed in mitochondria [15]. Thus a mitochondrial transporter carrying cis-aconitate across the mitochondrial membrane appears to be important in the biosynthesis of itaconic acid. The importance of this transporter has been demonstrated by van der Straat et al. which have developed an *A. niger* strain capable of producing itaconic acid, employing the natural capacity of this fungus to secrete very high amounts of citric acid which shares with itaconate many steps of its biosynthetic pathway [16]. These authors introduced the *A. terreus* itaconic acid

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biosynthetic cluster, consisting of the gene encoding for cis-aconitate decarboxylase, a mitochondrial transporter and a plasma membrane transporter, into *A. niger*. In particular, the only expression of a codon optimized cis-aconitate decarboxylase led to a low producing strain. The additional expression of the mitochondrial transporter gene resulted in an over 25-fold increased secretion of itaconic acid, pinpointing the importance of this mitochondrial transport activity for the itaconic acid production. Although the gene encoding this MC is known, the biochemical properties of this transporter have not been studied. These studies would be useful to further increase the production of itaconic acid.

MCs might be of importance also in a new approach of metabolic engineering, the so called *subcellular metabolic engineering*, where an entire metabolic pathway is compartmentalized to mitochondria in order to increase the production of interest [17]. This approach reproduces the natural strategy of the eukaryotic cells (and rarely of prokaryotic) of creating confined regions (organelles and metabolosomes) which offer a more efficient way to increase metabolic fluxes [18]. Pathways that are naturally cytoplasmic might benefit from mitochondrial compartmentalization, because the confinement of enzymes and metabolites to subcellular compartments may result not only in an increase in their local concentrations but also in the ability to reduce the toxicity of pathway intermediates, bypass inhibitory regulatory networks or avoid competing pathways [17]. Thus, *subcellular metabolic engineering* has the potential to provide multiple mechanisms to improve the performance of engineered pathways. Recently *subcellular metabolic engineering* has been successfully applied to improve the production of branched-chain alcohols in yeast, by combining overexpression and mitochondrial targeting of the complete isobutanol biosynthetic pathway [17].

The use of mitochondrion as an intracellular reactor for the production of bulk or fine chemicals can further increase the importance of MCs as rate controllers of the exchange of substrate and products in and out of mitochondria. Indeed, it is important to avoid bottlenecks in the traffic across inner mitochondrial membrane that can limit the metabolic flow of interest. In the previously cited production of branched-chain alcohols, pyruvate enters mitochondria where alcohols are produced and exported from. It is tempting to speculate that the whole process might be further improved increasing the pyruvate uptake into mitochondria. Very recently the proteins responsible for this biological activity have been identified [19,20]. In particular, in yeast three genes encoding the subunits of the pyruvate carrier, *MPC1*, *MPC2* and *MPC3*, have been identified. Hence, their overexpression could increase the flux of pyruvate into mitochondria. This hypothesis remains to be tested.

In conclusion, compartmentalization and the regulation of metabolic fluxes by controlling the expression of mitochondrial transporters, enabling to increase the substrate uptake in the mitochondrial matrix and the product export in the cytoplasm, can represent a crucial tool for metabolic engineering and strain development. More research is needed to determine to what extent metabolic fluxes are modified by changing of the expression level of specific MCs and to apply this knowledge to improve relevant biotechnological processes.

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