Biochemistry of microbial itaconic acid production

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INTRODUCTION

Itaconic acid (2-methylidenebutanedioic acid) is an unsaturated dicarbonic acid which has a high potential as a biochemical building block, because it can be used as a monomer for the production of a plethora of products including resins, plastics, paints, and synthetic fibers. Some Aspergillus species, like A. itaconicus and A. terreus, show the ability to synthesize this organic acid and A. terreus can secrete significant amounts to the media (>80 g/L). However, compared with the citric acid production process (titters >200 g/L) the achieved titers are still low and the overall process is expensive because purified substrates are required for optimal productivity. Itaconic acid is formed by the enzymatic activity of a cis-aconitate decarboxylase (CadA) encoded by the cadA gene in A. terreus. Cloning of the cadA gene into the citric acid producing fungus A. niger showed that it is possible to produce itaconic acid also in a different host organism. This review will describe the current status and recent advances in the understanding of the molecular processes leading to the biotechnological production of itaconic acid.

Keywords: cis-aconitic acid decarboxylase, Aspergillus terreus, Aspergillus niger, metabolic engineering, biochemical pathways, microbial organic acid production, industrial microbiology.

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Acid was detected in mammalian cells, where it was found in the formation of itaconic acid in nature. Very recently, itaconic acid was identified in mammalian cells.

But, up to now no specific gene encoding this enzymatic activity as a cis-aconitate transport is speculative. Besides A. terreus, itaconic acid is known to be produced also by other fungi like U. zune (Haskins et al., 1955), U. maydis (Haskins et al., 1955; Klement et al., 2012), Cancillula sp. (Tabuchi et al., 1981), and Rhodotorula sp. (Kawamura et al., 1981). No further investigations exist about the underlying reaction principles leading to itaconic acid formation in those species. However, recent evidence (Streit et al., 2011; Voll et al., 2012) points into the direction that CadA activity constitutes the general pathway toward the formation of itaconic acid in nature. Very recently, itaconic acid was detected in mammalian cells, where it was found in the cytosolic fraction. The proposed mechanism is that cis-aconitate is transported via the malate–citrate antiporter into the cytosol. Besides A. terreus, itaconic acid makes use of the mitochondrial malate–citrate antiporter or uses another mitochondrial carrier protein to be translocated to the cytosol.

Acid is known to inhibit isoctirate lyase (Williams et al., 1971; McFadden and Purohit, 1977), which is the crucial part of the glyoxylate shunt, and thus can act as an antibacterial agent. On the other hand, itaconic acid can inhibit fructose-6-phosphate 2-kinase (Sakai et al., 2004) and thus have a direct influence on the central carbon metabolism. In rats it was shown that an itaconate diet leads to a reduced visceral fat accumulation, because of a suppressed glycolytic flux (Sakai et al., 2004).

**ITACONIC ACID PATHWAY SPECIFIC ENZYMES AND GENES**

The reaction catalyzed by the cis-aconitic acid decarboxylase was already described in 1957 (Bentley and Thiessen, 1957a,b). Subsequently performed 14C and 34C labeling experiments (Winiskill, 1983; Bonnarme et al., 1995) confirmed the reaction scheme depicted in Figure 2. Itaconic acid is formed by an aliphatic rearrangement and decarboxylation from cis-aconitic acid removing either carbon C1 or C5 from the starting citric acid molecule (because of the symmetry of the molecule).

Furthermore, certain properties of the A. terreus CadA enzyme were determined: it has a $K_m$ value of 2.45 mM (37°C, pH 6.2) and a pH optimum of 6.2 ( Dwarti et al., 2002). At pH 7.5 the activity drops significantly and is below 20% of the maximal value ( Dwarti et al., 2002). Until 2008, the sequence of the CadA protein was unknown, because the protein exhibits a general low stability. Kanamasa et al. (2008) were able to purify a substantial amount of the enzyme. By sequencing of the protein the N-terminal and four internal sequences were determined, which produced a single hit, ATEG_09971, in the genome database of A. terreus. The gene was named cad and its protein product CadA. However, according to the nomenclature guidelines for Apiceralbusi it should rather be named cadA and CadA. The activity of the enzyme as a cis-aconitic acid decarboxylase (IC 4.1.1.6) was confirmed after heterologous expression of the gene in Saccharomyces cerevisiae. The CadA protein is a 490 amino acid protein (35 kDa) and has a high sequence identity with proteins from the MmgE/PpyPpP family, which includes 2-methylcitrate dehydratases. However, it is not clear, whether CadA has also a 2-methylcitrate dehydratase activity or whether a family member of the MmgE/PpyPpP class has also an activity as a cis-aconitic acid decarboxylase.

In contrast to the enzyme purification strategy, Li et al. (2011) used a transcriptomic approach to identify the cadA gene. A clone of the A. terreus strain NRRL1960 was cultivated at different conditions (pH, dissolved oxygen, etc.), which yielded different productivities and titers for itaconic acid. The conditions, which exhibited the highest difference in productivity and titer, were transcriptionally analyzed on a microarray with the assumption that genes involved in the itaconic acid pathway show an altered (higher) expression level during producing conditions. The cadA gene was highly scored in this analysis and thus can be identified in such an analysis. Interestingly another gene, encoding a mitochondrial carrier protein, was also highly scored in this analysis. This gene is located directly upstream of the cadA gene on the genome in A. terreus. Downstream of the cadA gene another transporter can be found which is annotated as a putative Major Facilitator Superfamily transporter. The mitochondrial carrier protein was detected in the transcriptomic analysis and was shown to have a direct positive influence on the itaconic acid production (Joer et al., 2011;...
FIGURE 2 | In the citric acid cycle cis-aconitic acid is formed as an intermediate during the conversion of citric acid to isocitric acid. cis-aconitic acid is decarboxylated by the CadA enzyme to itaconic acid releasing CO₂ (Bentley and Thiessen, 1957b).

van der Straat et al., 2012). However, the mechanism and substrates of this putative transporter are still unknown and its role needs to be clarified, but it can be speculated that intermediates of the biosynthesis pathway like cis-aconitic acid are transported with this protein.

The activity of the cis-aconitic acid decarboxylase is crucial for the performance of the whole itaconic acid biosynthesis pathway. In an itaconic acid overproducing strain, which was obtained by an selection on high itaconate levels (Yahiro et al., 1995), five times higher transcription levels of the cadA gene were found than in a comparable wild type strain but no change in the amino acid sequence was detected (Kanamasa et al., 2008). Expressing the cadA gene in A. niger under various constitutive promoters of different expression strength demonstrated that the itaconic acid productivity directly correlates with the cadA transcript level (Blumhoff et al., 2013). It can be concluded that a high transcriptional level of this gene is essential for an optimal production performance. A high transcriptional level of the gene might be necessary, because of a low stability of the enzyme in vivo, which was found to be rather unstable in vitro (Dwiarti et al., 2002; Kanamasa et al., 2008).

CATABOLIZATION OF ITACONIC ACID

Much is known about the biosynthesis of itaconic acid and the underlying enzymatic mechanisms, but for a complete biochemical picture of a certain metabolite, also the knowledge about its degradation is necessary. Unfortunately, the information about the degradation pathway of itaconic acid is scarce. In mammalian cells (guinea pig and rat liver) it was found that itaconate is converted to itaconyl-CoA (Adler et al., 1957) and is further processed via

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When the \textit{cadA} gene of \textit{A. terreus} (Tevz et al., 2010) was placed under the control of the \textit{gpdA} gene in \textit{A. niger}, the isomerase became possible. Li et al. (2011) expressed the \textit{pfkA} gene, which is already known to support the production of high titers of itaconic acid, in \textit{A. niger}. This level is not comparable with current existing strains or targeted genetic engineering. The unique and crucial step in the biosynthesis pathway is the decarboxylation of aconitic acid to itaconic acid. As already mentioned, the expression of this gene leads to an increased itaconic acid production. Furthermore, the strains exhibited a better recovery after the aeration was interrupted (Lin et al., 2011). This goal could be reached by further breeding of currently existing strains or targeted genetic engineering.

In \textit{A. terreus}, a gene was shown to influence the performance of itaconic acid production, which is a key enzyme of glycolysis. A. terreus currently limited to about 85 g/L. Although this is already a substantial amount it cannot be compared with the production of citric acid where titers over 220 g/L are steadily obtained in industrial processes. Transferred to the itaconic acid production in \textit{A. terreus}, a gene was shown to exhibit a higher activity than the \textit{gpdA} gene. Indeed, the expression of this gene leads to an increased itaconic acid production. Furthermore, the strains exhibited a better recovery after the aeration was interrupted (Lin et al., 2004).

There is the possibility that the genetic make-up of \textit{A. terreus} is not efficient enough to support the production of higher titers of organic acids. Therefore, a strategy is to genetically engineer the itaconic acid biosynthesis pathway into another host organism, which is already known to support the production of high titers of organic acids. As already mentioned, \textit{A. niger} is such a candidate. The unique and crucial step in the biosynthesis pathway is the decarboxylation of cis-aconitic acid toward itaconic acid. When the \textit{cadA} gene was placed under the control of the \textit{gpdA} gene, the isomerase became possible. Li et al. (2011) expressed the \textit{cadA} gene in \textit{A. niger} strain AB 1.13. For this purpose, the \textit{cadA} gene was placed under the control of the \textit{gpdA} gene. This enabled a strong and constitutive expression. An \textit{A. niger} strain which expresses the \textit{cadA} gene alone has the ability to produce about 9.7 g/L itaconic acid. This level is not comparable with current production strains of \textit{A. terreus}, but is a promising point for further engineering steps. Further attempts to rise the yield are to express genes like the above mentioned mitochondrial carrier protein together with the \textit{cadA} gene (Jure et al., 2011; van der Straat et al., 2012).

**OUTLOOK**

Iaconic acid as a renewable organic acid is of growing interest for the chemical industry, because of its potential to replace crude oil based products like acrylic acid. Up to now, the microorganism-based processes were improved by classical strain breeding and optimizations of the fermentation strategies and conditions. Especially the knowledge about the biotechnological process including oxygen supply, media compositions, and different bioreactor systems was significantly expanded (Kaerz et al., 2012). Regarding the media composition, it was found that copper ions positively influence the itaconic acid production in a genetically engineered \textit{A. niger} strain (Li et al., 2012). However, it is not understood which biochemical reactions are responsible or involved in such an effect. As already mentioned above, the biochemical reactions and effects of itaconic acid in the production hosts are not fully described. The catabolization pathway of itaconic acid requires further investigations in order to engineer a production host with a disabled degradation pathway. The effect of itaconic acid on other metabolic pathways is also of interest because the understanding of its physiological role can prevent undesired side effects (toxicity, health risk, pathway inhibition) and increase the safety of its use. Furthermore, it can be an interesting target for medical research because in mammalian cells it was detected in a metastatic tumor cell line (Strelko et al., 2011). Further knowledge about its role as an enzyme inhibitor can help to develop less-resistant enzyme varieties like in the case of the phosphofructokinase 2. Another target for further engineering is the CadA enzyme, which is described as an unstable protein. Prolonging its stability in vivo can help to increase the efficiency of existing production hosts. Also the genetic regulation of the itaconic acid pathway in \textit{A. terreus} requires a profound analysis. Li et al. (2011) have shown that genes involved in the biosynthesis pathway (\textit{cadA}) can be identified by transcriptomic approaches. However, nothing is known so far about the regulatory mechanisms leading to the expression of those genes.

The investigations on the molecular principles of itaconic acid synthesis revealed that cis-aconitic acid decarboxylase is the dedicated step in its biosynthesis in \textit{A. terreus}. Genetic engineering of this enzymatic step also renders other microbial hosts like \textit{A. niger} to producers of itaconic acid.

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