Amino acid transport in Penicillium chrysogenum in relation to precursor supply for beta-lactam production
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Chapter 6

Summary and concluding remarks

Summary

Amino acid transport in *Penicillium chrysogenum*. Fungi are capable of accumulating amino acids from their environment and use them as nitrogen and/or carbon source or as building blocks for protein or peptide synthesis. Amino acid uptake is mediated by a large set of permeases that differ in substrate specificity and regulation. Transport mostly occurs as proton symport and is thus is driven by the proton gradient (78, 181). Nearly all amino acid permeases identified so far comprise one family, named the AAP (amino acid permease) family (6). This family is distinct from two distantly related methionine/cysteine permeases of *Saccharomyces cerevisiae* (Mup1 and Mup2 (90)), and the unrelated Mtr type permeases of *Neurospora crassa* and *Penicillium chrysogenum* (46, 190) (see below). The AAP family and Mup1 and Mup2 are subdivided in the large and ubiquitous family of the amino acid/polyamine/organocation (APC) secondary transporters (91). The number of AAPs varies between organisms, and is 18 in *S. cerevisiae* (6, 91), approximately 20 in *Aspergillus nidulans* and 8 in *Ustilago maydis* (Trip et al, unpublished work). The relatively large number of amino acid permeases enables cells to fine tune specificity and selectivity to the differing environments in order to transport the preferred amino acids from a complex mixture.

Based on physiological and kinetic studies, nine different amino acid transport systems were identified in *Penicillium chrysogenum* (82). However, this number may be an underestimation due to overlapping substrates specificities and different regulation patterns. Since *P. chrysogenum* is closely related to *A. nidulans*, it may contain a similar large number of AAPs for which the function remains to be elucidated. In this thesis work, three amino acid permeases belonging to the AAP family and a fourth amino acid permease unrelated to AAPs have been cloned and characterized by means of functional expression in *S. cerevisiae* M4276. This strain lacks Gap1, the main general amino acid permease and Dip5, the acidic amino acid permease. Therefore, it shows a dramatically reduced ability to transport most amino
acids, while it is defective in the uptake of acidic amino acids. ArlP was cloned by means of a degenerated primer strategy which was based on conserved sequence motifs in fungal AAP transport systems. ArlP is specific for aromatic amino acids and leucine which it transports with moderate affinity \( (K_m, 350 \ \mu \text{M}) \) (209). PcDip5 is the acidic amino acid permease. It is homologous to Dip5 of \textit{S. cerevisiae}, and was cloned after complementation of the previously mentioned \textit{S. cerevisiae} strain M4276. Dip5 transports glutamate and aspartate with high affinity \( (K_m, 35 \ \mu \text{M}) \) while \( \alpha \)-aminoadipate is recognized with low affinity \( (K_m, 800 \ \mu \text{M}) \). PcGap1, the general amino acid permease which is homologous to Gap1 of \textit{S. cerevisiae} and that transports all amino acids, was cloned using a degenerated primer strategy based on regions specifically conserved in (putative) fungal general amino acid permeases (208). A fourth permease, PcMtr, was cloned after complementation of citrulline uptake in \textit{S. cerevisiae} M4276. This cloning strategy was expected to yield the general amino acid permease, but instead a system was obtained that is homologous to Mtr of \textit{Neurospora crassa}. Both transport aromatic and neutral aliphatic amino acids. They are not members of the APC family, but of the auxin and amino acid permease (AAAP) family (231), comprising members that vary strongly in amino acid sequence, but share a common topology of 11 transmembrane segments, instead of the typical 12 transmembrane segment topology of most APC transporters (91, 231).

**Uptake of \( \alpha \)-aminoadipate uptake.** The acidic amino acid \( \alpha \)-aminoadipate is one of the three amino acid precursors of penicillin, together with cysteine and valine. In the cytosol, they are condensed by ACV synthetase to form the tripeptide L-\( \alpha \)-aminoadipyl-L-cysteinyl-D-valine (ACV), which is transformed into isopenicillin N (IPN) by IPN synthase. IPN enters the microbodies by a so far undefined transport mechanism whereupon the \( \alpha \)-aminoadipate moiety is replaced by phenylacetic acid to form penicillin G. The latter is secreted by the cell. The intracellular concentration of \( \alpha \)-aminoadipate, but not of cysteine and valine (92, 115) appears limiting for the rate of ACV formation and therefore also for the overall penicillin synthesis rate. \( \alpha \)-aminoadipate is an intermediate of the lysine biosynthesis pathway, but is also channeled into the penicillin biosynthesis pathway. The intracellular concentration of \( \alpha \)-aminoadipate can be increased by blocking the enzymatic step that leads \( \alpha \)-aminoadipate conversion in the lysine route (26), or by the external addition of \( \alpha \)-
aminoadipate (59, 81). This leads to increased levels of penicillin production. Uptake of \( \alpha \)-aminoadipate is mediated by the acidic amino acid permease PcDip5, as well as the general amino acid permease PcGap1 (this thesis). PcDip5 transports glutamate and aspartate with high affinity \((K_m, 35 \, \mu M)\) whereas \( \alpha \)-aminoadipate is transported with a lower affinity only \((K_m, 800 \, \mu M)\). The general amino acid permease transports all amino acids and shows a somewhat higher affinity for \( \alpha \)-aminoadipate than PcDip5 \((K_m, 230 \, \mu M)\). Both transporters transport \( \alpha \)-aminoadipate only in the L-configuration. \( \alpha \)-Aminoadipate transport via PcGap1 is strongly inhibited (approx. 95%) by a ten-fold excess of leucine, whereas PcDip5 mediated transport is barely affected by the presence of leucine. This phenomenon was used to discriminate between PcGap1-and PcDip5-mediated \( \alpha \)-aminoadipate uptake in high penicillin yielding \( P. \) chrysogenum strains. When \( \alpha \)-aminoadipate is added at 25 \( \mu M \) to penicillin producing mycelium, PcGap1 and PcDip5 contribute almost equally to the uptake of \( \alpha \)-aminoadipate.

The activity of amino acid transporters in fungi is regulated at the transcriptional and post-transcriptional level. In this work, the expression of the \( PcGAP1 \) and \( PcDIP5 \) genes in \( P. \) chrysogenum growing in media with different nitrogen and carbon sources was tested by semi-quantitative RT-PCR. Expression of \( PcGAP1 \) was low when ammonia was present, while expression was high when glutamate or urea was used as sole nitrogen source. \( PcDIP5 \) showed a similar expression pattern, although \( PcGAP1 \), and not \( PcDIP5 \), was moderately expressed when serine, a substrate of PcGap1 and not of PcDip5, was the sole nitrogen source. In penicillin producing medium which contains urea and glutamate as nitrogen source and lactose as carbon source, expression of \( PcGAP1 \) and \( PcDIP5 \) was at an intermediate level. So, both \( PcGAP1 \) and \( PcDIP5 \) seem to be under nitrogen catabolite repression (NCR) and are expressed when cells are grown in the presence of urea (a poor nitrogen source) or glutamate as sole nitrogen source (this thesis).

\section*{AAP family permeases and Mtr homologs.} With the availability of an increasing number of completed fungal genome sequences (most of them ascomycetes), the abundance of AAPs and Mtr homologs in fungi, could be investigated. Usually, a distinct group of AAPs was found in each fungus, varying in number from 8 in \( U. \) maydis to approximately 20 in \( A. \) nidulans. In an evolutionary
tree, AAPs appear to cluster in groups specific for basic amino acids, acidic amino acids, and proline. This family also contains less well defined groups of general amino acid permeases and permeases specific for neutral amino acids (this thesis). In most filamentous fungi, one clear, distinct Mtr homolog (with more than 50% amino acid identity) is present, with the exception of *Fusarium graminearum*, which has 5 Mtr homologs (this thesis). The physiological role of these Mtr homologs remains to be elucidated. However, rather than play a general role in amino acid uptake, these systems may fulfill a function in the uptake of signaling molecules during differentiation. It will be of interest to test this hypothesis by the selective deletion of Mtr transporters in fungi. On the other hand, a *N. crassa* mutant in which the *mtr* gene was inactivated showed a strongly reduced uptake level of neutral amino acids when the cells were grown on minimal medium with ammonia as nitrogen source (190). This would suggest that Mtr fulfills a role in amino acid uptake, although a role in the uptake of signaling molecules under more physiological conditions cannot be excluded.

**Random mutagenesis of PcDip5.** The AAP family of transporters has not been studied in detail. Therefore, little is known about the structure/function relationships in AAPs nor is it clear which residues contribute to the binding sites to determine substrate selectivity. Alignments of AAPs show the presence of strongly conserved regions and residues, while some regions seem to be specifically conserved in AAPs with similar substrate specificity as for instance for the arginine (160) and a proline permease (201). However, none of these residues seem to be directly involved in substrate selectivity or binding. We have undertaken an attempt to use random mutagenesis and selection to improve the affinity or capacity of PcDip5 for α-aminoadipate transport. A special constructed strain of *S. cerevisiae*, able to utilize α-aminoadipate as primary nitrogen source but lacking the transporter to take it up from the medium, was transformed with a mutant library of *PcDIP5* and over 100,000 transformants were spread on plates in which a low concentration of α-aminoadipate was limiting for growth. Colonies of larger size were further analyzed, but none of the clones showed an increased α-aminoadipate transport. Apparently, this selection procedure which makes use of colony size is insufficiently reliable to perform such a screening. As more AAP family members are being characterized, amino acids positions that are conserved specifically among a group of transporters with similar
substrate specificity may lead to a better definition of possible amino acid targets which can be modified by site-directed mutagenesis approach. This may lead to the identification of residues that are directly involved in substrate binding and selectivity.

**Biotechnological application of the $\alpha$-aminoadipate transporter.** In the last decade, the study on the structure and functioning of non-ribosomal peptide synthetases, such as ACV synthetase, has made major progress (24, 108). In different fungi, a number of non-ribosomal peptide synthetases are found some of which are involved in the formation of a special class of peptides named peptaibols (31). These are helical peptides varying in length from 5 to 20 amino acids of which generally a large proportion is made up by $\alpha$-aminoisobutyric acid residues (Aib), from which the name peptaibol was derived. These peptides were found to have different interesting effects - often antibiotic activity against bacteria and fungi, but also neuroleptic effects in mice (169) – probably due to their membrane disturbing effect by the formation of oligomeric ion-channel assemblies (31). Non-ribosomal peptide synthetases consist of a series of linked modules, in which each module is responsible for the recognition, activation and incorporation of one residues into the peptide chain (161, 229). A potential application of these peptide synthetases is to recombining modules by genetic engineering, thereby creating new peptides with novel properties. The possibility of recombining the modules has been investigated in the European project Eurofung (for designing and improving health- and food-related production processes using filamentous fungal cell factories) using ACV synthetase of *Penicillium chrysogenum* as a model system. The replacement of the $\alpha$-aminoadipate specific module by a new module, possibly originating from another organism and specific for a different (natural or unnatural) amino acid, could theoretically generate new tripeptides. In order to introduce unnatural amino acids, efficient production of such peptides would require exogenous availability of such compounds. By means of active uptake a sufficiently high intracellular concentration may be obtained in order to obtain an effective peptide synthesis. In most cases, the substrate will not passively diffuses across the cytoplasmic membrane, and because of its broad substrate specificity, engineered variants of the general amino acid permease may provide a mechanism to allow entry of such compounds into the cell.
One substrate suggested for a recombinant ACV synthetase is pipecolic acid. It is a six-carbon cyclic imino acid and serves as a substrate for some non-ribosomal peptide and polyketide synthetases in for example *Streptomyces hygroscopicus* (144). The pipecolic acid binding and activating unit that is involved in the synthesis of the immunosuppressant immunomycin in *S. hygroscopicus* may replace the α-amino adipate specific module of ACV synthetase in *P. chrysogenum*. By this strategy, a new synthetase may be generated that catalyzes the formation of a pipecolyl-cysteinyl-valine tripeptide. For this purpose, pipecolic should be present in the cytosol at a sufficiently high concentration. In *P. chrysogenum*, pipecolic acid is derived from the lysine biosynthesis pathway. It can be formed from α-amino adipate in three consecutive steps (137, 138). In a saccharopine reductase knock-out mutant strain (SR), lysine biosynthesis is abolished and pipecolic acid accumulates intracellularly. Through the external supply of α-amino adipate, the production of pipecolic acid in this strain could be further increased (138). Feeding may be more effective when an α-amino adipate transporter like PcDip5 or PcGap1 is overexpressed. For this purpose, the *PcDIP5* and *PcGAP1* genes were cloned behind the strong pcbC promoter. These constructs were transformed into a wild-type, low penicillin producing *P. chrysogenum* strain, and in the case of PcDip5, a moderate increase in uptake of α-amino adipate was observed (H. Trip, unpublished data). The effect on pipecolic acid formation in a saccharopine reductase knock-out strain is under investigation by the group of Prof. Martín (Léon, Spain). In this respect, an increased production of pipecolic acid is also of interest because it is a precursor for the indolizidine alkaloid swansonine, produced by *Metarhizium anisopliae*. This compound exhibits anti-tumor activity (42, 84) motivating the development of efficient production strategies.

**Concluding remarks**

Amino acid transporters from *P. chrysogenum* can be functionally expressed and characterized with respect to substrate specificity and kinetics in *S. cerevisiae*. The uptake of α-amino adipate in *P. chrysogenum* via PcDip5 and PcGap1 could be assayed and their individual contribution to α-amino adipate uptake in high yielding *P. chrysogenum* strains was determined using selective inhibition of PcGap1 by leucine. The exact role of these transporters during penicillin production, however, remains to
be established. As α-amino adipate is derived from the lysine biosynthesis pathway, the transporters may play a role in the maintenance of a high intracellular concentration of α-amino adipate, i.e., as to prevent cellular leakage of this compound. However, to address this question, a single and double, \( PcDIP5/PcGAP1 \) knock-out mutant of \( P. \ chrysogenum \) needs to be constructed. Although this was attempted, the technical difficulty and low efficiency of homologous recombination in \( P. \ chrysogenum \) currently hampers such a genetic analysis.
Chapter 6