L-Phenylalanine and methanol metabolism in the facultative methylotroph Amycolatopsis methanolica (Nocardia sp. 239)
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Microorganisms growing on one-carbon substrates possess unique pathways for the generation of energy and for conversion of C1 units into intermediates of central metabolism. The further synthesis of cell constituents such as polysaccharides, proteins, nucleic acids and lipids occurs via the established pathways of intermediary metabolism, as found in other organisms. Current studies in our laboratory focus on the regulation of C1 metabolism in autotrophic bacteria (Calvin or RuBP pathway), methylotrophic bacteria (RuMP pathway) and methylotrophic yeasts (XuMP pathway).

Methanol is a commodity chemical of high purity. It is an attractive feedstock for fermentation processes which aim to produce bulk chemicals. There is also a strong interest in applying the unique metabolic pathways in methylotrophs to open up new, or alternative ways for the production of fine chemicals, such as amino acids. Methanol-utilizing bacteria employing the RuMP pathway are potential vehicles for fermentative overproduction of aromatic amino acids. The precursors for the shikimate pathway, erythrose-4-phosphate (E4P) and phosphoenolpyruvate (PEP), are intermediate and end product, respectively of the RuMP pathway. The Gram-negative RuMP cycle bacteria investigated, however, are obligate methylotrophs and not amenable to the extensive physiological and genetical manipulations required for strain development. The only currently available methanol-utilizing facultative RuMP cycle bacterium investigated, however, is A. methanolica, a Gram-positive bacterium belonging to the order Actinomycetales. The regulation of aromatic amino acid biosynthesis (and degradation) and methanol utilization in this organism constitutes the main topic of investigation in this thesis.

The increasing demand for L-phenylalanine as one of the building blocks of the dipeptide sweetener aspartame has initiated many investigations with the purpose to develop processes for L-phenylalanine production by whole cells or purified enzymes. High L-phenylalanine productivities were obtained from molasses and glucose using (poly)auxotrophic and deregulated strains of Escherichia coli, Corynebacterium glutamicum and Brevibacterium lactoferrum. An alternative approach is the use of (immobilized) enzymes or cells for conversion of suitable precursors into L-phenylalanine (Chapter 1). The industrial application of the latter processes for large scale L-phenylalanine production, however, is still limited.

A. methanolica is a very versatile bacterium, amongst others able to grow on L-phenylalanine and L-tyrosine as sole sources of carbon, energy and nitrogen. The catabolism of the amino acids proceeds via (4-hydroxy)-phenylpyruvate and (4-hydroxy)phenylacetate and the pathways merge at the level of homogentisate. The deamination of L-phenylalanine and L-tyrosine is catalyzed by an inducible NAD-dependent L-phenylalanine dehydrogenase and L-tyrosine aminotransferase, respectively. Following diepoxyoctane treatment, mutants blocked in either L-phenylalanine dehydrogenase or phenylpyruvate decarboxylase were isolated. These mutants, however, were still able to grow on L-phenylalanine albeit at strongly reduced growth rates. Only double mutants blocked in both enzymes completely failed to catabolize L-phenylalanine (Chapter 2).

The enzymatic production of L-phenylalanine with L-phenylalanine dehydrogenase has drawn considerable attention. The properties of this enzyme and the regulation of its synthesis were studied in more detail. Growth of wild type A. methanolica on D-phenylalanine involved an inducible amino acid racemase. The L-phenylalanine produced was further metabolized via phenylpyruvate, catalyzed by the NAD-dependent L-phenylalanine dehydrogenase. The latter enzyme was only detectable during growth on D- and L-phenylalanine and highest enzyme activities were already obtained at relatively low substrate concentrations. The presence of additional substrates resulted in decreased levels of L-phenylalanine dehydrogenase. In D- and L-phenylalanine-limited chemostat cultures the rate of enzyme
synthesis increased with increasing dilution (growth) rates. Growth on mixtures of L-phenylalanine plus methanol in batch cultures resulted in simultaneous utilization of both substrates. Methanol utilization did not repress L-phenylalanine dehydrogenase synthesis. Chemostat culture experiments revealed that enzyme production rates increased with increasing L-phenylalanine/methanol ratios in the medium reservoir. Purification of the enzyme from D- and L-phenylalanine-grown cells resulted in the isolation of enzymes with identical properties. The native enzyme consisted of a single subunit with a molecular weight of 42,000. The enzyme showed activity with L-phenylalanine, L-leucine, phenylpyruvate, 4-hydroxyphenylpyruvate, indole-3-pyruvate and \( \alpha \)-ketoisocaproic acid, but not with imidazolepyruvate, D-phenylalanine and other amino acids tested. Maximum activities with phenylpyruvate (310 \( \mu \)mol min\(^{-1}\) mg\(^{-1}\)) were estimated at pH 10 and 53°C. Sorbitol and glycerol stabilized the enzyme. The properties of the enzyme are compared with those of similar enzymes obtained from other sources (Chapters 1 and 3).

The mechanisms controlling aromatic amino acid biosynthesis are described in Chapter 4. The first enzyme involved in aromatic amino acid biosynthesis is 3-deoxy-D-arabino-heptulosonate 7-phosphate (DAHP) synthase. The presence of DAHP synthase isoenzymes frequently has been reported in other organisms. Only a single enzyme species could be detected in A. methanolicus. This enzyme was sensitive to cumulative feedback inhibition by the three aromatic amino acids. Partially purified enzyme showed apparent \( K_i \) values of 3, 160 and 180 \( \mu \)M for L-tryptophan, L-phenylalanine and L-tyrosine, respectively. The aromatic amino acids displayed competitive inhibition with respect to E4P. L-Tryptophan and E4P showed uncompetitive and competitive inhibition towards PEP, with apparent \( K_i \) values of 11 and 530 \( \mu \)M, respectively. Chorismate mutase functions in L-phenylalanine and L-tyrosine biosynthesis. In cell-free extracts the activity of this enzyme was inhibited both by L-phenylalanine and L-tyrosine (apparent \( K_i \) values of 60 and 35 \( \mu \)M, respectively). The activity of prephenate dehydratase, an enzyme specifically involved in L-phenylalanine biosynthesis, was inhibited by L-phenylalanine (apparent \( K_i \) value of 10 \( \mu \)M) and stimulated by L-tyrosine (activator constant of 10 \( \mu \)M). Anthranilate synthase, the first enzyme in the L-tryptophan specific branch, was strongly inhibited by L-tryptophan (apparent \( K_i \) value of 5 \( \mu \)M). Addition of the aromatic amino acids, either separately or in combinations, did not result in significant repression of the synthesis of these enzymes. Future work, aiming to achieve overproduction of L-phenylalanine by A. methanolicus, initially should focus on the isolation of mutant strains deregulated in feedback inhibition of DAHP synthase, chorismate mutase and prephenate dehydratase.

Further steps required for overproduction of aromatic amino acids by a L-phenylalanine dehydrogenase and phenylpyruvate decarboxylase minus double mutant of A. methanolicus (Noc 87-13) were studied in Chapter 5. A number of analogs of the aromatic amino acids were identified that showed strong inhibitory effects on the activities of regulatory enzymes in the biosynthetic pathway and growth of the wild type organism in glucose-mineral medium. Analogs of L-tryptophan especially inhibited DAHP synthase activity; L-phenylalanine- and L-tyrosine analogs especially inhibited chorismate mutase and prephenate dehydratase, and chorismate mutase, respectively. The growth inhibitory effects of a number of these amino acid analogs was reversed readily by the addition of the aromatic amino acids and/or their intracellular precursors phenylpyruvate, chorismate and anthranilate to the medium. The data indicate that ortho- and para-fluoro-D,L-phenylalanine and L-phenylalanine amide are the most suitable analogs for the isolation of mutants with feedback inhibition insensitive prephenate dehydratase. Attempts to isolate tyrosine and tryptophan auxotrophic mutants were only successful in the latter case and this resulted in the selection of a stable anthranilate synthase-negative mutant (Noc 87-13-14). The data obtained further suggest that uptake of aromatic amino acids in A. methanolicus is mediated by a common transport system. This necessitates the use of anthranilate, rather than L-tryptophan, as a supplement in future attempts to isolate L-tyrosine auxotrophic and amino acid analog resistant mutant strains of Noc 87-13-14.
The regulation of methanol metabolism in *A. methanolica* is described in Chapter 6. Mixed substrate experiments in batch cultures with glucose or acetate plus methanol resulted in simultaneous utilization of the substrates. The presence of glucose, but not of acetate, repressed synthesis of the RuMP cycle enzymes HPS and HPI, and methanol was only utilized as an energy source. Similar results were found following addition of formaldehyde (fed-batch system) to a culture growing on glucose. It is concluded that the synthesis of enzymes involved in methanol dissimilation and assimilation in *A. methanolica* is regulated differently. Methanol and/or formaldehyde induce the synthesis of these enzymes, but under carbon-excess conditions their inducing effect on HPS and HPI synthesis is overridden completely by glucose. Repression of HPS and HPI was of minor significance following addition of methanol to glucose-, acetate- and ethanol-limited chemostat cultures. Addition of a pulse of glucose to a formaldehyde-limited (2.5 mmol l\(^{-1}\) h\(^{-1}\)) fed-batch culture resulted in a decrease in the levels of several enzymes of methanol metabolism (including HPI), whereas the HPS levels remained relatively constant. A relative increase in the ratios of HPS and HPI activities was also observed with decreasing growth rates in formaldehyde-limited chemostat cultures. These observations indicate that additional mechanisms are involved in controlling the levels of these C\(_1\)-specific enzymes in *A. methanolica*. The strong repressive effects of some "heterotrophic" substrates on methanol metabolism makes it unlikely that the presence of methanol as an additional substrate will enhance the intracellular availability of E4P and PEP, precursors for aromatic amino acid biosynthesis. Nevertheless, utilization of methanol as an energy source may still allow a further increase in the flow of glucose-carbon towards biosynthetic rather than energy-generating processes. An alternative approach would be the isolation of mutants constitutively expressing HPS and HPI.

The organism used in the present studies was initially labelled *Streptomyces* sp. 239, then *Nocardia* sp. 239. Recent developments in the taxonomy of actinomycetes allowed its proper identification. Chemosystematic studies showed that the organism has a wall chemotype IV (meso-diaminopimelic acid, arabinose and galactose present). However, unlike representatives of the genus *Nocardia*, cell walls of the organism are devoid of mycolic acids. Further chemotaxonomic and morphological data, and a comparison of reverse transcriptase sequences of 16S ribosomal ribonucleic acid, identified the organism as a member of the genus *Amycolatopsis*. On the basis of a variety of biochemical and microbiological tests it was concluded that the organism forms the nucleus of a new species. Its reclassification as *Amycolatopsis methanolica* has been proposed (Chapter 7).