Enzymology and regulation of the atropine metabolism in pseudomonas putida

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INTRODUCTION

The breakdown of a chemical compound in living organisms is accomplished usually by a sequence of enzyme catalyzed reactions. As a result, such a compound is broken down by a fixed pattern, the metabolic pathway. In most cases, the cells in an organism are able to control the synthesis and activity of the enzymes involved. In this way, the cells are able to fine tune the capacity of a metabolic pathway to the actual demand.

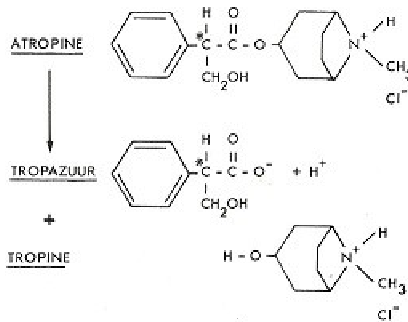
This PhD thesis research deals with the metabolism of the alkaloid atropine, in the bacterium Pseudomonas putida. It includes a study of the metabolic pathway of atropine in the bacterium and the enzymes involved. In addition, a special mechanism has been elucidated by which the regulation of this metabolic activity is accomplished.

Atropine is an alkaloid present in Nature a.o in the fruits of the plant Atropa belladonna L. and in other members of the Solanaceae family. The toxicity of extracts of atropa fruits is known within living memory (Hippocrates 400 BC); the active principle in these extracts was associated with the fate goddess Atropos who terminated life by cutting the thread of life.

The toxic action of atropine is based on the inhibition of transmission of nerve impulses in the parasympathetic nervous sytem. The compound has a number of important pharmacological applications that make use of this specific action. These comprise its action as antidotum in case of poisoning with organophosphorous compounds including many insecticides like parathion and chemical warfare agents.

The Medical Biological Laboratory (MBL) in the Dutch TNO Organization for Defense Research is carrying out a wide spectrum of projects to collect relevant data on the physiological activities of atropine. In this context, a study has been started on the interaction of atropine with its physiological receptor, this is the target structure for atropine. The binding of atropine to its receptor results in the disturbance of the impulse transmission in the central nervous system. So far, biochemical research of this receptor is not possible since the receptor can be recognized in vivo only. For this reason, a project has been started to find a more accessible biological structure, that shows a specific interaction with atropine and might be used as a kind of a model for the atropine sensitive receptor.

Literature data describe an enzyme present in rabbit serum, which can hydrolyze the ester atropine in the acid component tropic acid and the alcohol component tropine:



This enzyme is a rather aspecific esterase. Moreover, it is hardly to manage for enzymological research due to the low atropine esterase activity in Dutch rabbits.

A better study object became available when Rörsch and Berends isolated a bacterium from soil between the roots of Atropa belladonna (Berends et al 1969). This bacterium was able to grow in a synthetic medium with atropine as sole source of carbon. It was given name of PMBL-1 and has been identified by Bartlema and Wensinck as a Pseudomonas putida biotype A (Wensinck 1969). Rörsch and Berends were able to show the presence of an atropine-esterase in PMBL-1 grown with atropine as sole carbon source. In addition, they demonstrated the specificity of this bacterial enzyme for atropine and its suitability as an object of enzymological study.

This atropine esterase appeared to be inhibited by various compounds of the organophosphorus type like dïisopropyl phosphorofluoridate (DFP). This gave support to the idea that the esterase belonged to a group of enzymes – the so-called serine-esterases - which are inhibited by organophosphates. It has been shown that this inhibition is caused by the reaction of the organophosphate with a highly active serine residue that is present in the catalytic center of the enzyme.

Serine-esterases have been subject of intensive study a.o. in the MBL during the last 20 years (J.A. Cohen, R.A. Oosterbaan, H.S. Jansz and F. Berends, 1959). The reaction of the organophosphate with the serine residue can be used to investigate the mechanism of action of these serine-esterases. These studies have made important contributions to our present understanding of enzymatic hydrolysis.

The atropine esterase in Pseudomonas PMBL-1 seemed to be interesting as a good model for the atropine-sensitive receptor and as a new representative of the serine esterases. This because of its specificity for atropine and its sensitivity for inhibiting organophosphorus compounds. The bacterial origin of the enzyme might offer the additional advantage to isolate mutants of the bacterium which produce an atropine esterase with small modifications in the protein molecule. The study of the effects of these modifications on the interaction of atropine with atropine-esterase and on the mechanism of action of this enzyme could provide important information on these processes.

The investigation of the metabolic pathway of atropine was started initially with the intention to isolate mutants with a modified atropine esterase. In this respect it was important to know the metabolic pathway of atropine in Pseudomonas in order to distinguish between mutants with a modification by mutation in the genetic information coding for the esterase versus mutants blocked further down in the pathway.

Soon, it became obvious that the search of the metabolism of atropine offered a quite different perspective. The synthesis of atropine esterase is - as discovered by Rörsch and Berends - subject to control: the atropine esterase cannot be detected in Pseudomonas grown with sole carbon sources like glucose or succinic acid. Apparently, the atropine esterase is an inducible enzyme, its synthesis dependent on the presence of a specific ingredient present in the cultivation medium.

During the investigation of the ability of the hydrolysis products tropic acid and tropine to act as sole carbon source, both compounds were metabolized by Pseudomonas. Growth on tropic acid appeared to result in the synthesis of atropine esterase whereas after growth on tropine the enzyme was absent. This is remarkable in view of the relation of the tropic acid to the enzyme. The induction of the esterase by its product tropic acid drew attention for the regulatory mechanism that controls the synthesis of the esterase. Therefore, it seemed of interest to elucidate the metabolism of tropic acid and to identify the enzymes involved. It might be that those enzymes could be induced together with the atropine esterase. A study of the induction of these enzymes could reveal the regulation of protein and enzyme synthesis in PMBL-1.

In addition, study of the further breakdown of tropic acid might contribute to our knowledge of microbial metabolism of aromatic acids with a branched side chain. Our knowledge of the metabolism of this class of compounds is still very poor.

These considerations have resulted in the investigation reported in this thesis on the metabolic pathway of atropine and the regulation of synthesis of the enzymes involved.

Chapter 1 of this thesis presents a summary of the literature available on the metabolism of atropine in higher animals, in plants and in micro-organisms. Chapter 2 describes materials, methods and techniques used in this study whereas chapter 3 deals with the synthesis, identification and the keto-enol tautomerism of 2-phenylmalonic semi-aldehyde, a compound that in this study appeared to be one of the intermediary metabolites in the breakdown of atropine.

In chapter 4 experiments are discussed that presented the first evidence for the metabolism of atropine and tropic acid in Pseudomonas PMBL-1. Studies were carried out on the adaptation of PMBL-1 during growth on tropic acid and various other aromatic carbonic acids. Pseudomonas cultivated in a medium with atropine or tropic acid appeared to be adapted for growth on phenylacetic acid. The role for phenylacetic acid in the metabolism of atropine and tropic acid was confirmed by the isolation of phenylacetic acid from the growth medium of a few mutants with a block somewhere in the breakdown of tropic acid.

These data were the basis for a working hypothesis: conversion of atropine in phenylacetic acid is accomplished by hydrolysis in tropic acid followed by two dehydrogenations and one decarboxylation (see annex 1). The chapters 5 through 8 describe the investigations to confirm this working hypothesis and the identification of the enzymes involved in PMBL-1 in the conversion of atropine in phenylacetic acid.

The breakdown of atropine proceeds through tropic acid, 2-phenylmalonic semi-aldehyde and phenylacetaldehyde catalyzed by the enzymes atropine esterase (AtrE), tropic acid dehydrogenase (TDH), 2-phenylmalonic semi-aldehyde decarboxylase (PDC) and phenylacetaldehyde dehydrogenase (PDH). Together these enzymes are called the “tropic acid enzymes”. The identification, the quantitative assay, the partial purification and some properties of each of these enzymes will be presented. These enzymes are proteins enzymologically well to handle with a large specificity for the corresponding substrate.

In case of atropine esterase (chapter5) a purification procedure has become available that allows an enrichment on protein basis of 600x compared with the enzyme in a crude extract. In this way an allmost pure enzyme preparation has become available for further investigation of the catalytic center of this enzyme.

Chapter 6 describes tropic acid dehydrogenase (TDH). This enzyme catalyzes the dehydrogenation of tropic acid into 2-phenylmalonic semi-aldehyde (pma) by transfer of 2 hydrogens on the cofactor NAD^+. The study of the kinetics of the dehydrogenation has shown that this process is reversible in the presence of purified enzyme:

Establishment of the equilibrium was demonstrated both by dehydrogenation of tropic acid as well as by hydrogenation of pma. The slow production of NADH, that can be observed once the equilibrium has been reached, has been attributed to the shift of the equilibrium by spontaneous decomposition of pma.

Effect of keto-enol tautomerism of pma on the kinetics of the hydrogenation of pma and the position of the equilibrium has been studied in detail. The time course of the hydrogenation can be explained quantitatively on basis of the specificity of the tropic acid dehydrogenase for the keto form of pma and the rate of the tautomeric rearrangement enol-pma → keto-pma in aqueous condition. Although keto-pma not has been identified as the direct conversion product, the presented experimental evidence leaves no space for a conclusion other than keto pma being the direct metabolite of tropic acid.

PMBL-1 appears to possess a pma decarboxylase. This in spite of the Instability of keto-pma, what results in the spontaneous decarboxylation. Chapter 7 present the evidence for the presence of this enzyme, obtained by the study of a protein fraction that accelerates the enzymatic dehydrogenation at neutral pH many times. The identification as 2-phenylmalonic semi-aldehyde decarboxylase is based on the direct effect of this protein on the stability of pma and the formation of phenylacetaldehyde and CO_2.

Phenylacetaldehyde dehydrogenase (chapter 8) is the 2nd dehydrogenase contributing to the metabolism of atropine and tropic acid. Similar like tropic acid dehydrogenase it uses NAD^+ as cofactor. The enzyme is much less stabile compared with the other tropic acid enzymes, but can be stabilized by a buffer solution of special composition. Using this buffer, it is possible to purify the enzyme partially and investigate several of its properties.

Chapter 9 regards the functional sequence of the tropic acid enzymes as well as the presumable absence of an active system (permease) for the uptake of tropic acid. The metabolism of tropic acid in PMBL-1 is compared with that of mandelic acid in Pseudomonas ATCC 12633.

Chapter 10 deals with the regulation of synthesis of the tropic acid enzymes. The 4 enzymes in PMB-1 appeared to be induced in the presence of atropine or tropic acid in the growth medium. In addition, a few other compounds have been demonstrated to induce these enzymes. In a detailed study of the induction process in mutants of PMBL-1 evidence has been obtained that only phenylacetaldehyde and benzaldehyde induce the tropic acid enzymes that atropine and tropic acid have only the ability to do so if these compounds can be metabolized in phenylacetaldehyde. The apparent advantages of induction by

phenylacetaldehyde over the induction by atropine and tropic acid will be discussed.

The relevance of the elucidation of the tropic enzymes to the receptor model and to the mechanism of action of the atropine esterase will be discussed in the epilogue. Arguments will be presented for the hypothesis that the tropic acid enzymes are involved exclusively in the breakdown of atropine and probably not in a more central function in Pseudomonas. For this reason these enzymes might be very suitable for the further study of regulation of protein synthesis in general.