Enzymology and regulation of the atropine metabolism in pseudomonas putida

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CHAPTER 9

THE METABOLIC PATHWAY OF ATROPINE

9.1 INTRODUCTION

The tropic acid enzymes have been identified as the biocatalysts involved in the breakdown of atropine into phenylacetic acid in Pseudomonas PMBL-1. These enzymes have been described in the preceding chapters. This chapter deals with the mutual relationships of these enzymes in the atropine metabolism. The possibility is then discussed that - in addition to these – other enzymes are also induced especially for the metabolism of atropine. Indications will be discussed that the metabolism of atropine in other Pseudomonas PMBL strains proceeds according to the same metabolic pathway as in PMBL-1. Finally, the tropic enzymes will be compared with the enzymes involved in the breakdown of mandelic acid in Pseudomonas putida ATCC 12633.

9.2 SEQUENCE OF ACTION OF THE TROPIC ACID ENZYMES

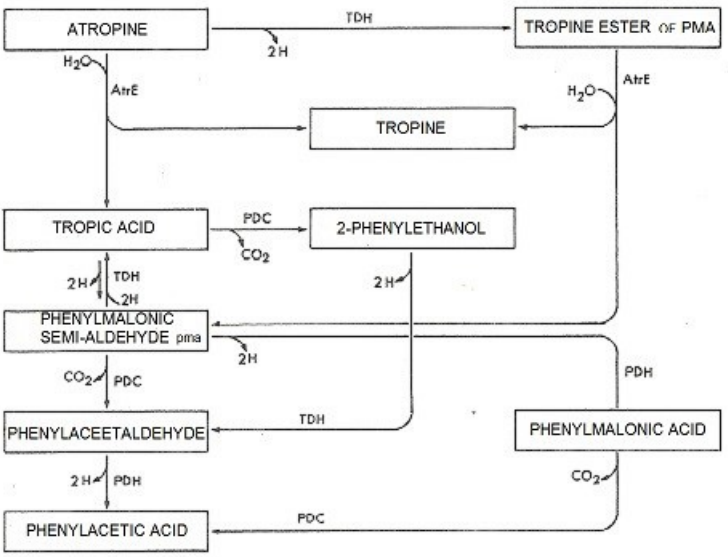
The metabolic pathway of atropine into phenylacetic acid (annex 1) is based on the substrate specificity of the tropic acid enzymes and on the identification of the products formed by these enzymes.

One could question whether these four tropic acid enzymes might operate in another sequence with the same final result. Other relevant other options are visualized in fig 9.1.

A first option is atropine as substrate for TDH and the formation of the tropine ester of pma. This compound might be converted by AtrE in pma and tropine. This option can be excluded since atropine cannot be dehydrogenated by TDH. This has been shown in the following experiment: The AtrE still present in a purified TDH sample was inhibited completely by treatment with the organophosphate Soman (pinacolyl methylphosphonofluoridate). The dehydrogenating capacity of this sample was tested with atropine, tropic acid and the methyl ester of tropic acid as substrate and compared with that of the same TDH sample not treated with Soman. The results presented in table 9.2 show clearly that neither atropine, nor the methyl ester of tropic acid were dehydrogenated by TDH. This also excluded other reaction schemes that form tropine ester of pma as first product.

Fig 9.1

Alternatives for breakdown of atropine into phenylacetic acid by the tropic acid enzymes.



A second possibility, the breakdown of tropic acid into phenylacetaldehyde through 2-phenylethanol can be excluded as well. The formation of CO_2 was not observed during the incubation of tropic acid with PDC, or with an extract of PMBL-1 cultivated in the presence of tropic acid (7.5.1). The compound 2-phenylethanol cannot act as a substrate for TDH and PDH (6.4.3). This compound is not suitable as carbon source (4.2) whereas the tropic acid enzymes were not induced in bacteria grown in a mix of succinic acid and 2-phenylethanol (10.3).

A third possibility is the conversion of pma by PDH in phenylmalonic acid, followed by a decomposition by PDC. The latter reaction however, did not happen; it was shown by thin layer chromatography that PDC is not able to convert phenylmalonic acid in phenylacetic acid (7.5). In addition, growth of PMBL-1 in the presence of phenylmalonic acid does not result in the induction of the tropic acid enzymes (10.3). Moreover, pma was not dehydrogenated by PDH (8.5).

On basis of these data, the conclusion is justified that breakdown of atropine occurs according to the scheme in annex 1 and not according to an alternative metabolic pathway.

Table 9.2

Substrate	Tropic acid dehydrogenase activity (U/ml) without Soman	0.2 mM Soman
Atropine	0.205	0.005
Methyl ester of tropic acid	0.120	0.000
Tropic acid	0.565	0.615

Atropine, the methyl ester of tropic acid and tropic acid as substrate for TDH in absence and presence of 0.2 mM Soman; THD activity assayed according to 2.9.3

9.3 OTHER ENZYMES POSSIBLY INVOLVED IN THE BREAKDOWN OF ATROPINE

9.3.1 Breakdown of tropine

Tropine is formed during hydrolysis of atropine by AtrE. It is obvious to check that tropine, which is produced during the hydrolysis of atropine by AtrE, will also be utilized by the bacterium. A clear indication is the usefulness of tropine as sole carbon source by PMBL-1. Moreover, tropine is not accumulated during the growth with atropine as carbon source, as appeared in the analysis of the growth medium using thin layer chromatography.

Niemer and Bucherer (1961) found in Corynebacterium “belladonnae” tropine to be dehydrogenated by an enzyme that uses NAD^+ as hydrogen acceptor. PMBL-1 cultivated in the presence of atropine was found to contain a tropine dehydrogenase which, however, transfers the hydrogen to the cofactor $NADP^+$.

This enzyme is absent in an extract of Pseudomonas grown with tropic acid as carbon source. Moreover, this dehydrogenase is apparently independent of the tropic acid enzymes and has not been studied in detail.

9.3.2 Uptake of substrate from the medium

Uptake of compounds by bacteria from a medium is often an active process. In a number of cases, transport proteins have been identified for the uptake of, amongst others, sulfate and sugars. For a recent overview see Pardee (1968).

Active transport of metabolites can be investigated by measuring the uptake of radioactivity if the compound with a radioactive label is presented to the bacterium. This has been carried out for Pseudomonas using ^3H-tropic acid, as described in 2.8. Wild type PMBL-1 cultivated in the presence of tropic acid

Table 9.3 Uptake of tritium by PBML-1 and mutants

Pseudomonas	Lacking enzyme	Medium	^{3}H-uptake
PMBL-1	—	A	3090 dpm
PMBL-1	—	B	210 dpm
PMBL-1	—	A	3090 dpm
PMBL-174	TDH	A	0 dpm
PMBL-176	TDH	A	575 dpm
PMBL-194	PDC	A	137 dpm
PMBL-192	PDC	A	805 dpm

Bacteria (1 ml; A_{700} = 1.5), cultivated in synthetic medium with 0.1% tropic acid + 0.1% phenylglyoxylic acid (A) or with 0.1% phenylacetic acid + 0.1 % phenylglyoxylic acid (B) were incubated with 1.6 x 10^5 dpm tropic acid during 60 min. The recorded radioactivity was corrected for the uptake by a control non- incubated culture (250-350 dpm) and is the average of 2 experiments.

(Ps-tropic acid) of phenylacetic acid (Ps phenylacetic acid) was incubated with ^{3}H-tropic acid. The amount of radioactivity taken up was measured in disintegrations per min (dpm) after 60 min (table 9.3). Ps-tropic acid appeared to take up tritium, Ps-phenylacetic acid did not. This could mean that Ps tropic acid has a transport system for tropic acid. The difference could also be explained by the fact that Ps tropic acid was metabolically adapted to tropic acid and thus could accumulate and convert this compound, resulting in an uptake of radioactivity.

In order to distinguish between these possibilities, the uptake of ^{3}H-tropic acid was assessed in 4 mutants, which could not produce either TDH or PDC anymore (10.7) as the result of mutagenic treatment. Since only one of the tropic acid enzymes was absent in these mutants, it was reasonable to expect that the damage brought about was limited. It seemed unlikely that in each of these mutants the information for a possible transport protein was lost as well.

These mutants were cultivated in a synthetic medium supplied with 0.1% tropic acid and 0.1% phenylglyoxylic acid as carbon source and thereafter incubated with the ^{3}H-tropic acid. The mutants showed only 0-25% of the uptake observed for the wild type under comparable conditions (table 9.3). These results make it is unlikely that a special transport system is in place for the uptake of tropic acid in Pseudomonas. This uptake is most probable a consequence of the capacity to convert tropic acid, since the uptake of tritium by the wild type coincides with an active metabolism of this compound.

The possibility cannot be ruled out that the bacterium has an uptake system at its disposal for atropine. However, similar experiments could not be carried out due to the insufficient radiochemical purity of the available ^{3}H-atropine.

9.3.3. Racemase

Pseudomonas fluid cultures with 0.05% (±) atropine as the sole source of carbon reached about the same optical density as with 0.05% (-) atropine. Apparently both optical isomers were metabolized. Since AtrE has a strong preference for (-) atropine, it does not seem unreasonable to expect a role of an atropine-racemase in the metabolism of the (+) isomer.

This enzyme should be present in an extract of Ps-atropine but not in an allmost pure sample of AtrE. One would expect the hydrolysis of (+) atropine could be more rapidly in the presence of the extract compared with hydrolysis by the purified enzyme. Such a difference was not observed. Therefore, it is unlikely that a racemizing enzyme is involved in the breakdown of the (+) enantiomer.

Although the bacterium can utilize both (-) and (+) tropic acid as sole source of carbon, it does not seem necessary to suppose the presence of a tropic acid racemase. TDH does not show a clear preference for either of the stereo-isomers of tropic acid. This is remarkable in view of the large substrate specificity of the enzyme (6.4).

This inconsistency could be overcome if tropic acid during the enzyme action (at pH 9.5) would racemize spontaneously. This was investigated using a polarimeter. The sponta neous racemizing appears to proceed too slowly to explain the dehydrogenation of (+) tropic acid by a stereospecific TDH. It would also be possible that a tropic acid racemase exists and is still present in the TDH samples used. This cannot be excluded in advance because the TDH is only partially purified in the method described (6.3). The racemization of (+) and (-) tropic acid in the presence of purified TDH has been investigated by polarimetry. Supporting evidence was not obtained however. It is therefore unlikely that a racemase is involved in the dehydrogenation of (+) or (-) tropic acid.

The discrepancy between the observed substrate specificity and lack of stereo specificity remains unexplained.

9.4 BREAKDOWN OF ATROPINE IN OTHER PMBL STRAINS

Rörsch (personal communication) has isolated in addition to PMBL-1 eight other micro-organisms from soil, able to metabolize atropine. These strains have

Table 9.4

Specific activity of the tropic acid enzymes in a number of Pseudomonas strains

Strain	Classified as	AtrE	TDH	PDC	PDH
PMBL-1	putida biotype A	2.2	2.2	1.9	0.22
PMBL-2001	putida biotype A	±0.6	± 1.0	0.77	± 0.3
PMBL-4001		3.6	0.73	0.57	0.09
PMBL-4501	putida biotype A	1.3	1.1	1.1	0.15
PMBL-5001		3.0	0.65	0.51	0.10
PMBL-5501	putida biotype B	+ 1.6	0.35	0.25	0.07
PMBL-6001		3.0	0.67	0.50	0.13
PMBL-6501		± 1.1	0.51	0.75	0.07
PMBL-7001	fluorescens	2.3	0.82	1.0	± 0.4

The strains were cultivated in synthetic medium with 0.1% tropic acid and 0.1% succinic acid. The specific activity is assayed in the extract according to 2.10 and reported as the number of Units per mg protein.

been deposited in the collection of the Medical Biological Laboratory under the PMBL numbers 2001, 4001, 5001, 5501, 6001, 6501 and 7001. Five of these strains have been classified by Wensinck (1969) as Pseudomonas putida or fluorescens, the other strains cannot be classified under the existing types but are definitely Pseudomonaceae.

The strains have been cultivated in a synthetic medium with tropic acid and analyzed for the presence of AtrE, TDH, PDC and PDH activity. In table 9.4, the specific activities have been listed. The total activities are in line with these values. The strains PMBL-4001, PMBL-5001 and PMBL-6001 contain a relative high amount of AtrE. These esterase activities are inhibited by organophosphates. This holds for the other strains as well. The greatest activity of TDH and PDC was found in PMBL-1. In this comparison, one has to keep in mind that the reaction conditions for the enzyme assay have been set up for PMBL-1. These might be suboptimal for the corresponding enzymes in the other strains. It is therefore not certain that PMBL-1 has indeed the largest amount or the most active TDH and PDC.

9.5 RELATIONSHIP WITH THE BREAKDOWN OF MANDELIC ACID AND PHENYLPYRUVIC ACID

Stanier et al (1953) have isolated the bacterium Pseudomonas putida (ATCC 12633, biotype A), able to grow with mandelic acid as sole source of carbon. The breakdown of mandelic acid in this bacterium is elucidated by Stanier (1953), Gunsalus (1953 a and b), Hegeman (1966), Kennedy (1968) and their coworkers. The metabolic pathway of mandelic acid and the mandelic enzymes involved are summarized in fig 9.5. The pattern of breakdown shows a remarkable similarity with the metabolism of tropic acid in PMBL-1. One could ask oneself whether one or more tropic acid enzymes would be identical to the corresponding enzymes from the mandelic acid metabolism.

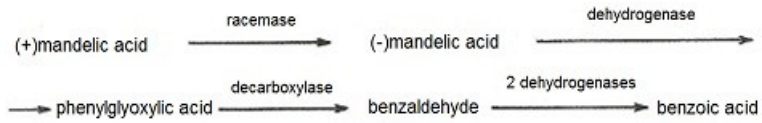


Fig 9.5 Metabolic pathway of mandelic acid in Pseudomonas ATCC 12633

This has been investigated. Pseudomonas ATCC 12633 was kindly provided by Dr. G.D.Hegeman (Berkeley, California) and deposited in the MBL strain collection under number PA-20, a notation used below.

The Pseudomonaceae PA-20 and PMBL-1 are not identical because PA-20 does not grow with tropic acid as sole source of carbon and PMBL-1 cannot metabolize mandelic acid (4.2). Moreover, it has been shown that none of the tropic acid enzymes is identical to one of the mandelic enzymes: mandelic acid, phenylglyoxylic acid and benzaldehyde are not converted by purified TDH, PDC and PDH respectively (see chapters 6 and 7 and 8). An extract of Ps tropic acid lacks the mandelic enzymes and Pseudomonas PA-20, cultivated with mandelic acid as sole carbon source, does not contain any of the tropic acid enzymes.

The difference between the metabolic pathways of mandelic acid and that of tropic acid not only concerns the substrate specificity of the enzymes involved. The equivalent for the AtrE is not present in the extract of PA-20. The hydrolysis of the methyl ester of mandelic acid is not catalyzed by the extract. Strain P-20 contains a racemase in contrast to PMBL-1. The mandelic dehydrogenase is stereospecific for (+) mandelic acid, bound to a particle fraction and sedimented by centrifugation. In vitro, the enzyme makes use of the dye 2,6-dichlorophenol-indophenol as hydrogen acceptor and does not transfer the hydrogen to the cofactor NAD^+. The phenylglyoxylic acid decarboxylase needs thiamine pyrophosphate as cofactor, whereas in dehydrogenation of benzaldehyde in PA20, 2 dehydrogenases are involved with NAD^+ and $NADP^+$ respectively as cofactors.

It is clear that the strains PA-20 and PMBL-1 have complete different enzyme systems for the breakdown of tropic acid and mandelic acid respectively.

PMBL-1 is not able to breakdown mandelic acid, but phenylglyoxylic acid is metabolized. In PMBL-1, this breakdown is analogous with the phenylglyoxylic acid metabolism in PA-20. The compound is converted by decarboxylation and dehydrogenation in benzoic acid (see annex 1).

The production of benzaldehyde by an extract of Pseudomonas PMBL-1 cultivated in the presence of phenylglyoxylic acid has been confirmed using thin layer chromatography. The pertaining decarboxylase has been demonstrated as well. This enzyme is not identical to PDC. A mutation that results in a loss of PDC has no effect on the ability to use phenylglyoxylic acid as carbon source (10.7). Reversely, PMBL-132 lacking the phenylglyoxylic acid decarboxylase and unable to metabolize phenylglyoxylic acid anymore, contained the PDC and was able to grow undisturbed with tropic acid as sole source of carbon.

In strain PMBL-1, benzaldehyde is converted into benzoic acid by a benzaldehyde dehydrogenase that needs $NADP^+$ as hydrogen acceptor. It cannot use NAD^+ for this purpose. This enzyme distinguishes itself from PDH which only can use NAD^+ as cofactor. The kinetics of the complete conversion of benzaldehyde at low substrate concentration leads to the conclusion that this enzyme has a very low Michaelis constant for this substrate. In this way, it looks like PDH. The low Michaelis constant has been used gratefully to develop an assay for phenylglyoxylic acid decarboxylase.

The benzaldehyde dehydrogenase is able to dehydrogenate small amounts of benzaldehyde nearly completely under production of the stoichiometric amount of NADH, thanks to the great affinity of the enzyme for this substrate. Therefore, this enzymatic conversion can be used for the quantitative assay of benzaldehyde, produced from phenylglyoxylic acid by decarboxylation.

As shown by Seidenberg (1962) and Asahawa (1968), phenylpyruvic acid is decarboxylated to phenylacetaldehyde and then oxidized to phenylacetic acid. This study was done in Achromobacter euridyce. The decarboxylase needs thiamine pyrophosphate and Mg^{2+} as cofactors. This enzyme is rather aspecific and takes care for the decarboxylation of indole derivatives as well. These authors did not study the dehydrogenation of phenylacetaldehyde in phenylacetic acid. In this organism, like in PMBL-1, phenylacetaldehyde is produced by enzymatic decarboxylation. There might be a relationship between the breakdown of

phenylpyruvic acid and the conversion of tropic acid. However, phenylpyruvic acid is not decarboxylated by PDC (7.5). Moreover, it appears that in mutants of PMBL-1, in which the PDC is lacking, the metabolism of phenylpyruvic acid is no disturbed. Apparently, PDC is not involved in the metabolism of phenylpyruvic acid.

The data show that in PMBL-1 different enzyme systems are available for the breakdown of tropic acid, phenylglyoxylic acid and phenylpyruvic acid. These data, especially those related to the enzymes phenylglyoxylic acid decarboxylase and the benzaldehyde dehydrogenase have been very valuable in the study of the synthesis of the tropic acid enzymes, described in the next chapter.