Enzymology and regulation of the atropine metabolism in pseudomonas putida

Stevens, W.F.

Summary

From soil between the roots of Atropa belladonna L., Rörsch and Berends (1965) isolated a Pseudomonas putida (PMBL-1) which is able to grow with the alkaloid atropine as sole carbon source. It has been shown that the breakdown of atropine in this micro-organism proceeds according to the scheme facing the last page. Atropine is hydrolyzed, tropine and tropic acid are formed. The latter is broken down via 2-phenylmalonic semi-aldehyde (pma) and phenylacetaldehyde to phenylacetic acid, a common carbon source to many Pseudomonas strains.

The role of phenylacetic acid as an intermediate in the metabolism of atropine and tropic acid was deduced from the observations that PMBL-1 grown on atropine or tropic acid is adapted to phenylacetic acid (fig. 4.2) and that all mutants isolated for the inability to metabolize phenylacetic acid fail to grow with atropine or tropic acid as carbon source. Moreover, phenylacetic acid was isolated from the growth-medium of several of these mutants grown in the presence of tropic acid.

The hydrolysis of atropine is catalyzed by the enzyme atropine-esterase (AtrE). The action of this esterase has been demonstrated by the identification of the reaction products tropine and tropic acid (fig. 5.2). This fairly stable enzyme has been purified extensively: estimated purity over 90% (table 5.1). The enzyme is specific for (-)atropine and closely related compounds (table 5.6) and is inhibited by organophosphates like diïsopropyl phosphorofluoridate (DFP).

The second enzyme playing a role in the metabolic pathway is the NAD^+ dependent tropic acid dehydrogenase TDH, the third is the 2-phenylmalonic semi-aldehyde decarboxylase (PDC). The combined action of the two enzymes on tropic acid in the presence of NAD^+ results in the formation of NADH (fig 6.3), phenylacetaldehyde (fig 7.4) and CO_2 (table 7.5). The last step is catalyzed by the phenylacetaldehyde dehydrogenase (PDH), also NAD^+ -dependent. It was shown that phenylacetic acid is the product of this reaction (fig. 8.4). The TDH, PDC and PDH have been purified partially (tables 6.2; 7.6; 8.3). For each enzyme, a reliable determination of enzyme activity has been developed (fig 2.2). The enzymes were found to be specific for the appropriate substrates (table 6.5.1 and 6.5.2).

When the dehydrogenation of tropic acid was studied in the presence of NAD^+ and purified TDH at neutral pH, a fast initial NADH-production was observed, followed by an abrupt slowing down (fig 6.6). This phenomenon was explained on the assumption that the enzyme catalyzes the equilibrium process:

$$\text{tropic acid} + NAD^+ \rightleftharpoons \text{pma} + NADH + H^+$$

The NADH concentration increases rapidly while the equilibrium is established;

the slow NADH-production thereafter is caused by spontaneous decomposition of pma, resulting in a shift of the equilibrium to the right. Variation of the concentrations of tropic acid and NAD^+, variation of the pH and the addition of NADH and pma all had the expected influence on the course of the reaction. The equilibrium was obtained either by dehydrogenation of tropic acid or by hydrogenation of pma (fig. 6.9). From the results, the equilibrium constant K_{eq} was calculated approximately to amount to 0.30×10^{-12} M (table 6.17); this means that the hydrogenation is favored.

The pma required for these experiments has been synthesized according to the only procedure described in literature (Strukov 1952). The melting point of the synthesized product was not the same as the one cited. The compound, however, was identified unquestionably as pma in its enol-form by means of chemical analysis and infrared spectroscopy.

The reaction of enol-pma with NADH in the presence of TDH also shows biphasic kinetics (fig. 6.11.a). Evidence is presented that the keto-pma is the proper substrate for the TDH; the initial rapid conversion of NADH into NAD^+ was found to correspond with the amount of keto-pma initially present. The slow phase of the reaction was attributed to a rate limiting tautomeric conversion of enol-pma into keto-pma.

The rate of decomposition of enol-pma and keto-pma was measured (fig 6. 15). These values were compared with the rate of reaction governing the NADH production after the establishment of the equilibrium. From these results can be concluded that keto-pma is the product formed during the enzymatic dehydrogenation of tropic acid.

When non-purified TDH preparations are used for the dehydrogenation of tropic acid, the equilibrium is not reached. This is shown to be the consequence of the pma-decarboxylase present in these preparations. The decarboxylase has a great effect on the stability of keto-pma. This enzyme can also be demonstrated by its great effect on the amounts of NADH (fig. 7.2), phenylacetaldehyde and CO2 formed after establishment of the equilibrium during enzymatic dehydrogenation of tropic acid at neutral pH. The effect on the NADH-production is used for a quantitative assay for the decarboxylase. During purification, the enzyme is separated from TDH (fig 7.1).

The phenylacetaldehyde dehydrogenase is less stable than the other enzymes. Much activity is lost during dialysis (table 8.1) and during storage of preparations with rather low protein content (table 8.2). In a phosphate buffer (0.05 M), containing mercapto-ethanol, EDTA, sodium-dodecylsulfate and phenylacetaldehyde, the enzyme can be handled without serious loss of activity.

The pathway from atropine to phenylacetic acid as given in the scheme is, owing to the substrate specificity of the four enzymes, the only possible route mediated by these enzymes. A quite different pathway seems unlikely since all mutants unable to grow with atropine as sole source of carbon lack one or more of these enzymes. Indications that PMBL-1 contains a permease or a racemase for atropine or tropic acid have not been found. The enzymes involved in the breakdown of atropine in PMBL-1 are quite different from the enzymes in Pseudomonas ATCC 12633 which are concerned with the metabolism of the structurally related mandelic acid (Hegeman 1966).

The AtrE, TDH, PDC and PDH are inducible enzymes. Growth in atropine and tropic acid containing media results in the induction of all four enzymes. Also phenylacetaldehyde, phenylglyoxylic acid and benzaldehyde act as inducers. (table 10.3 and 10.4). From studies of mutants of PMBL-1, with a mutation in only one of the genes for these enzymes, it was concluded that atropine and tropic acid themselves are unable to induce the “tropic acid enzymes” but have to be converted into the actual inducer phenylacetaldehyde (table 10.6). The same holds for the induction observed in PMBL-1 in phenylglyoxylic acid containing media; this compound is not a real inducer but must be converted into benzaldehyde by a decarboxylase present in PMBL-1 (table 10.7).

The advantages of induction of the enzymes by an intermediate product are discussed. This mode of induction is more specific than induction by the substrate (Ornston 1966): the compound has to be recognized by three specific enzymes before it can give rise to induction. Moreover, because of the relatively high basic activity of PDH, induction will only take place if atropine can be converted into phenylacetaldehyde in a really fast process. This enables the cell to check beforehand whether induction will be of advantage or not. This is very attractive from a regulatory point of view.

In the final discussion it is concluded that these enzymes present in PMBL-1 have the exclusive function to catalyze the breakdown of atropine into phenylacetic acid. These enzymes are therefore considered as a very promising object for a study of the regulation of protein synthesis in micro-organisms.