Enzymology and regulation of the atropine metabolism in pseudomonas putida

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CHAPTER 7

THE 2-PHENYLMALONIC SEMI-ALDEHYDE DECARBOXYLASE

7.1 INTRODUCTION

Third enzyme in Pseudomonas PMBL-1 involved in the conversion of atropine into phenylacetic acid is the 2-phenylmalonic semi-aldehyde decarboxylase (PDC). It is not possible to measure the activity of PDC in a simple way (for instance by measuring CO_2 production), because the decarboxylation of 2-phenylmalonic semi-aldehyde (keto-pma) takes place spontaneously in aqueous environment (half life time about 90 sec pH 7.5 25^0).

To measure the activity and to investigate the properties of this enzyme, use has been made of the effect of PDC on the enzymatic dehydrogenation of tropic acid in the presence of purified TDH in neutral environment. In this process, the rate of the NADH production after establishment of the equilibrium is determined by the speed of withdrawal of the keto-pma from the equilibrium. Since this decarboxylation is accelerated by the PDC, it is possible to use this NADH production as a measure for the activity of the decarboxylase.

This chapter deals with the identification of the enzyme (7.2), description of its assay (7.3), the partial purification (7.4) and properties (7.5) of the purified enzyme. In the discussion (7.6) the question is raised why an enzyme is present in Pseudomonas to catalyze a reaction that proceeds spontaneously very well.

7.2 INDICATIONS FOR THE EXISTENCE OF A DECARBOXYLASE

In the preceding chapter, it has been shown that the dehydrogenation of tropic acid by purified enzyme in neutral environment proceeds through the establishment of an equilibrium followed by a rate limiting non-enzymatic decarboxylation of the keto-pma formed.

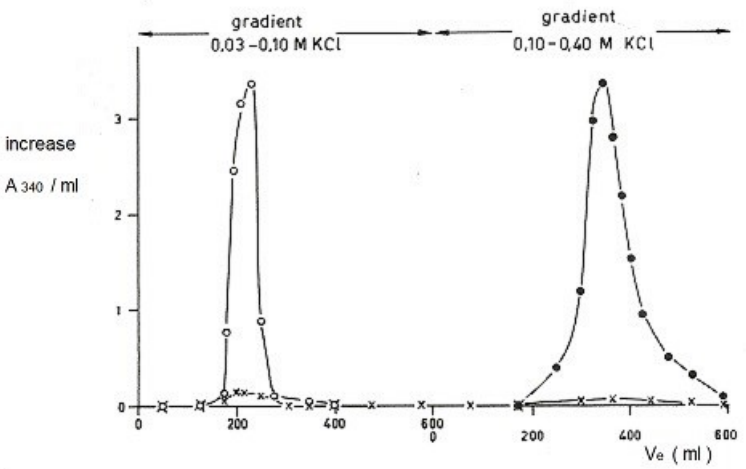
The establishment of the equilibrium was not observed using non-purified TDH samples. This suggested that in the crude TDH another component could be present able to accelerate the decarboxylation of keto-pma, so a terminating reaction would result instead of a slowly shifting equilibrium.

Chromatography of non-purified TDH

The establishment of the equilibrium was only noticed using TDH purified with DEAE cellulose chromatography. Therefore, it was conceivable that the presumed component mentioned above was separated from the TDH. This suggestion was confirmed in the following experiment.

A DEAE cellulose column was used to absorb a non-purified TDH sample. The column was eluted first with 600 ml HMP with an increasing salt concentration 0.03 - 0.1 M KCl and thereafter with 600 ml HMP with an increasing salt concentration of 0.1 - 0.4 M KCl (fig 7.1). The eluate was investigated for the presence of TDH with the activity assay at pH 7.5. Measured in this way, small activity x – x – x was present in fraction 40-50 (elution volume 200-250 ml).

Fig 7.1
Fractionation of a TDH sample on DEAE cellulose



The column was eluted with 600 ml salt gradient 0.03 - 0.1 M KCl in HMP (fraction 1-120) and subsequently with 600 ml 0.1 - 0.4 M KCl in HMP (fraction 121-240). On the ordinate: the TDH activity in the fractions (after adding to tropic acid and NAD^+ in 33 mm K-phosphate pH 7.5) as the total increase in absorption, during the first 3 min calculated for 1 ml eluate.
x – x – x increase absorption measured without addition
• – • – • increase of absorption measured with 50 µL fraction 46 added
o – o – o increase of absorption measured with 50 µL fraction 192 added
Fraction volume 5 ml. More data on chromatography please see chapter 2.11

Following the suggestion above, one could imagine that in the other fractions a component was present able to stimulate the NADH production by purified TDH at pH 7.5. The NADH production by TDH fraction 46 was tested for a possible effect on any of the other fractions. Curve x – x – x represents TDH activity without addition, curve • – • – • TDH activity of fractions in presence of fraction 46.

The fractions 160 – 240 (elution volume 200-600 of the second salt gradient) showed at pH 7.5 a much larger NADH production in the presence of fraction 46 than expected on basis of the fractions separately. In these combined fractions

different kinetics were observed as well: whereas with fraction 46 only NADH production quickly decreased (establishment of the equilibrium), fraction 46 showed in the presence of any of the fractions 160-240, kinetics of a continuous NADH production as it was observed with non-purified TDH preparations at this pH. The fractions 160-240 might contain a component able to withdraw the pma from the equilibrium.

In the same way, "fraction 192" was investigated. Named after the most active fraction, "fraction 192"is the collective name for the combined fractions 180-200 (100 ml; elution volume 300-400 of the second salt gradient); the effect of "fraction 192" was studied on the NADH production in the presence of fractions with TDH activity at pH 7.5. The NADH production by the fractions 40-50 was stimulated considerably by fraction 192. Also in this case the NADH production shifted from a biphasic process into a more continuous formation of NADH.

Samples of fraction 192 treated with proteolytic enzymes (subtilisin, pronase) or heated at 100^0 for 5 min lost their stimulating activity; therefore, the responsible component was most probably a protein.

Effect of fraction 192 on the equilibrium catalyzed by TDH

The change in kinetics is illustrated in fig 7.2. Purified THD taken from fraction 46 was incubated with tropic acid and NAD^+ at pH 7.5. After establishment of the equilibrium, 100 µL of fraction 192 was added. This resulted instantly in a NADH production with the kinetics of the non-purified enzyme.

A similar result was obtained by adding an amount of hydrazine instead of fraction 192. Hydrazine is known to react with aldehydes; it might be able to withdraw the pma from the equilibrium. The resemblance of the effect of the protein fraction and the hydrazine supports the assumption that the effect of fraction 192 has to be ascribed to a component that catalyzes conversion of pma.

Effect of fraction 192 on the stability of keto-pma

Direct proof that the protein in question catalyzes the conversion of pma – and not affects the enzymatic dehydrogenation in another way - was obtained by study of the effect of this protein on the stability of keto-pma; the enzymatic hydrogenation reported in 6.6 was used for the assay of keto-pma.

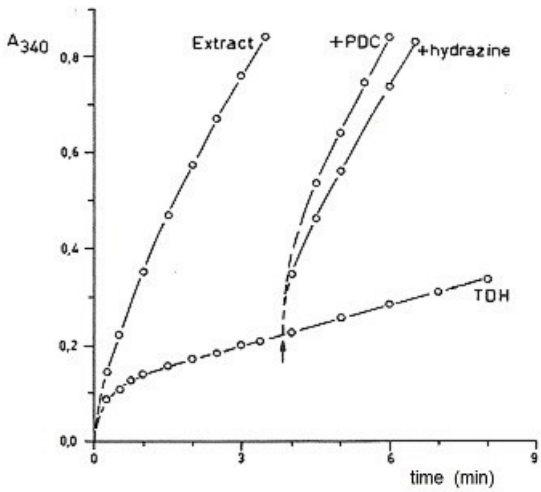
The effect of the protein on the stability of pma was assessed by quantifying the amount of pma that was decomposed in 33 mM K-phosphate pH 7.5 during 30 sec, compared with the amount of pma converted in the presence of fraction 192 under comparable circumstances.

The results reported in table 7.3 show that in the absence of the fraction 192

protein 21% of the pma is converted spontaneously after 30 sec. In the presence of the protein fraction 192, pma is converted completely in the same amount of time.

Fig 7.2

Effect of PDC and hydrazine on the enzymatic dehydrogenation of tropic acid at pH 8.0



Tropic acid and NAD^+ were incubated with non-purified TDH ("extract") or with purified TDH ("TDH" fraction 46 fig 7.1). After 3 min and 45 sec were added: 50 µL fraction 192 ("PDC") and 0.25 M hydrazine pH 8.0 respectively. Correction has been made for the change in volume. Further details see fig 6.6

Fig 7.3

Effect of the protein fraction on the stability of pma

incubation time (sec)	"fraction 192"	concentration pma mM
0	–	0.067
30	–	0.053
30	+	<0.001

Keto-pma (in ethanol-water 1-20) was incubated with 0,3 µmol NADH in 3 ml 33 mM K-phosphate pH 7.5 at 25^0 in absence or presence of 20 µL fraction 192. On time = 0 and time = 30 sec, the pma concentration was measured by addition of TDH (see 6.6).

It is obvious to suggest that the conversion of pma by the protein component, will be the decarboxylation of pma and the protein thus a decarboxylase. This has been investigated by analysis of the products formed.

Effect of fraction 192 on the production of phenylacetaldehyde

During the enzymatic decarboxylation one expects the formation of CO_2 and phenylacetaldehyde, but these compounds will also be formed spontaneously during the fast decarboxylation of keto-pma. If one wants to discriminate between enzymatic decarboxylation and this spontaneous decomposition, special precautions have to be taken. For instance, a very short incubation time with a large amount of enzyme, or a system which offers a low concentration of keto-pma to a relatively high amount of enzyme during a prolonged period of time. The latter was realized by incubating tropic acid, NAD^+ and TDH in the presence of a NADH → NAD^+ regenerating system: pyruvic acid as hydrogen acceptor and lactic acid dehydrogenase. This system converted the NADH formed under consumption of pyruvic acid in NAD^+ and lactic acid. The NADH concentration remained low. The dehydrogenation was not inhibited by the NADH formed (6.4.5 and 6.5).

The conversion of tropic acid in this system was investigated in presence and absence of fraction 192. In the presence of the fraction, a considerable amount of phenylacetaldehyde was formed during incubation at 25^0 during 60 min. This was visualized by thin layer chromatography, elution fluids BEM (fig 7.4), EMX.

In the absence of fraction 192, only a small amount of phenylacetaldehyde was formed (data not shown). This experiment proofs that the protein fraction is involved in the formation of the product of decarboxylation: phenylacetaldehyde. This conclusion in combination with the direct effect of the enzyme on keto-pma is a strong indication that the protein fraction 192 contains the 2-phenylmalonic semi-aldehyde decarboxylase (PDC).

The reaction product was identified by treatment of a diethyl ether extract of the incubation mixture with 2.4dinitrophenylhydrazine, followed by analysis by thin layer chromatography. The formation of the 2.4 dinitrophenylhydrazon of phenylacetaldehyde was demonstrated: with the elution fluid benzene: petroleum ether $40\text{-}60^0$ (75:25 v/v); the hydrazon of the product had a R_f value of 0.27; R_f of the hydrazon of reference compound phenylacetaldehyde 0.28.

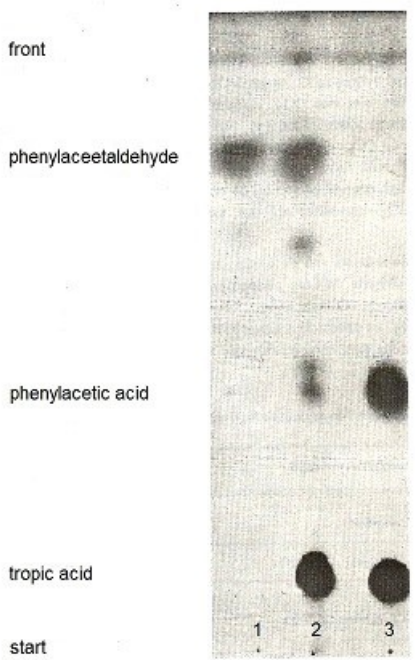
In gas liquid chromatography, the primary product of the conversion had a retention time of 8 min, equal to that of the reference phenylacetaldehyde.

Effect of fraction 192 on the production of CO_2

The formation of the reaction product CO_2 and the effect of fraction 192 on this formation was studied by the Warburg manometric method (2.6).

Fig 7.4

Thin layer chromatography of the incubation mixture of tropic acid, TDH and PDC



The reaction mixture consisted of tropic acid (300 µmol), NAD^+ (10 µmol), TDH 20 U and fraction 192 (1 ml) in 15 ml 0.1 M K-phosphate pH 8.5. A NAD^+ regenerating system (450 µmol pyruvic acid and 0.3 mg LDH) was added. Incubation at 25^0 during 60 min. The reaction mixture was extracted with diethyl ether and analyzed by chromatography with elution fluid BEM. Detection H_2SO_4/HNO_3

1. phenylacetaldehyde
2. incubation mixture
3. tropic acid and phenylacetic acid

Pyruvic acid (R_f 0.12) can not be made visible with the detection method used.

Tropic acid, NAD^+, TDH and fraction 192 were deposited in the main compartment of the Warburg vessel and incubated in the presence of the NAD^+ regenerating system. The incubation was carried out at pH 8.4. CO_2 if formed would remain as bicarbonate in solution. After 60 min, H_2SO_4 was added from the side compartment; from the subsequent change in pressure, the amount of µliter CO_2 formed during the incubation was calculated.

Table 7.5 shows the considerable amount of gas released from the complete incubation mixture. With KOH in the side compartment, there is no gas production; therefore, the developed gas is CO_2. The CO_2 production did not occur in the absence of TDH or fraction 192. The large effect of this fraction on the CO_2 production is a further confirmation of the role of this enzyme in the breakdown of tropic acid. Thiamine pyrophosphate and pyridoxal phosphate, common cofactors in enzymatic decarboxylation do not have an effect on the CO_2 development; there was no effect of Mg^{++} or EDTA.

This confirms the direct role of protein fraction 192 in the decarboxylation of pma into phenylacetaldehyde and CO_2. It concerns here the 2 phenylmalonic semi-aldehyde decarboxylase. By the identification of phenylacetaldehyde and CO_2 two products have been identified indirectly formed in the dehydrogenation of tropic acid.

Fig 7.5

CO_2 production during the conversion of tropic acid by TDH and PDC

Incubation mixture	mM	µL CO_2
complete	–	209
middle compartment with KOH	–	9
no TDH	–	4
no PDC (fraction 192)	–	6
+ thiamine pyrophosphate	0.15	195
+ pyridoxal phosphate	0.15	210
$MgCl_2$	0.5	219
EDTA	0.5	198

The incubation mixture in the main compartment of the Warburg mini vessel contained 13 µmol tropic acid, 1 µmol NAD^+, 0.8 U TDH, 0.2 ml fraction 192 and the NAD^+ regenerating system (0.3 mg LDH and 18 µmol pyruvic acid) in 2.7 ml 40 mM K-phosphate pH 8.4. In the inner compartment, 0.1 ml water or 0.1 ml 0.5 N KOH was deposited; in the side compartment, 0.2 ml 2 N H_2SO_4. After 60 min incubation at 30^0, the sulfuric acid was added and the CO_2 released measured.

7.3 THE QUANTITATIVE ASSAY OF THE PDC ACTIVITY

The assay of PDC (see 2.9.3) is based on the effect of this decarboxylase on the enzymatic dehydrogenation of tropic acid. The enzyme assay was carried out as follows: 10 mM tropic acid, 0.8 mM NAD^+ and 0.8 U TDH were incubated in phosphate buffer pH 8.5 until the rate of NADH production was controlled by the spontaneous decarboxylation of pma. Next, PDC was added and the further NADH production registered. The PDC activity was calculated from the difference of the NADH production before and after the addition of PDC.

To prevent that in the presence of PDC the shift in the equilibrium became rate controlling, excess TDH (0.8 U) was used. The tropic acid and NAD^+ concentration and the pH are based on the results of introductory experiments, in which these parameters were varied. At a higher pH or at a higher substrate or NAD^+ concentration is it not possible to measure the effect of PDC accurately due to the high absorption at 340 nm as the result of the establishment of the equilibrium under these conditions. Choosing for a lower pH or a reduced tropic acid or NAD^+ concentration lowers this absorption, but the increase of absorption registered after the addition of PDC was under these circumstances not directly proportional with the time, preventing an accurate assay of this enzyme.

The conditions described allow well for an accurate assay of PDC. The enzyme activity measured is directly proportional with the amount added (fig. 2.2).

This enzyme assay is not affected by TDH that might be present in PDC samples because this enzyme is already added in excess. But the PDC assay is disturbed by the next enzyme in the metabolic pathway of tropic acid, the phenylacetaldehyde dehydrogenase PDH. Under the conditions described, the product of the decarboxylation (phenylacetaldehyde) is converted in phenylacetic acid under production of NADH. This can be repressed by heating the enzyme sample (0.5 mg/ml) on 55^0 during 15 min; this results in complete inactivation of the PDH while the PDC keeps nearly its full activity.

7.4 PARTIAL PURIFICATION OF THE PDC

The result of the purification of the decarboxylase enzyme (2.11.2) is reported in table 7.6. The enzyme has been purified 85 x comparing with the crude bacterial extract. Yield 29%. The enzyme was nearly completely separated from the other tropic acid enzymes.

The behavior during gel filtration over Sephadex G-100 allows an estimation of the molecular weight on 45.000 (2.11.3); this treatment did not result in further purification.

Table 7.6

Partial purification of the PDC from PMBL-1

Preparation	TA	yield	SA	purification
Extract	7	100%	0.76	1x
After streptomycin precipitation	6.9	99%	–	–
After ammonium sulfate precipitation	3.9	56%	3.5	5x
After DEAE chromatography	2.0	29%	64.2	85x

Enzyme activity and protein concentration were measured according to 2.9. TA is reported in 10^3 U. SA in U/mg protein.

The isoelectric point of the enzyme is 4.6 as determined by electrophoresis in a gradient of LKB 'Ampholytes'.

7.5 PROPERTIES OF THE PURIFIED ENZYME

7.5.1 Substrate specificity

The assay for the activity of the PDC is based on the decarboxylation of the pma, formed as the result of the enzymatic dehydrogenation of tropic acid. Therefore, it is not possible to use this test (7.3) to study the PDC substrate specificity.

The ability of aromatic acids to serve as substrate for PDC has been investigated by incubating about 20 µmol of these compounds with the enzyme (2 U) at pH 7.0 and 25^0 during 1 hour. The incubation mixture was acidified and extracted with di-ethyl ether. The extract was analyzed for the presence of metabolites using thin layer chromatography with elution fluids EMX and BEM.

Detection by H_2SO_4-HNO_3, 2,4-dinitrophenylhydrazine and rhodamine B, an agent for universal detection of UV absorbing compounds. Incubation without PDC served as control.

The pma was decarboxylated into phenylacetaldehyde, both spontaneously and in the presence of PDC. However, none of the following compounds was decarboxylated by the PDC:

phenylglyoxylic acid	2-phenyl-2-hydroxypropionic acid
phenylpyruvic acid	phenylacetic acid
phenylmalonic acid	2-phenylpropionic acid
tropic acid	2-hydroxy-phenylacetic acid

In principle, this method can be used to show decarboxylation. This is concluded from similar experiments in which phenylglyoxylic acid was incubated with the phenylglyoxylic decarboxylase that can be isolated from Pseudomonas ATCC 12633 (9.5); in this case, a considerable conversion in benzaldehyde was demonstrated.

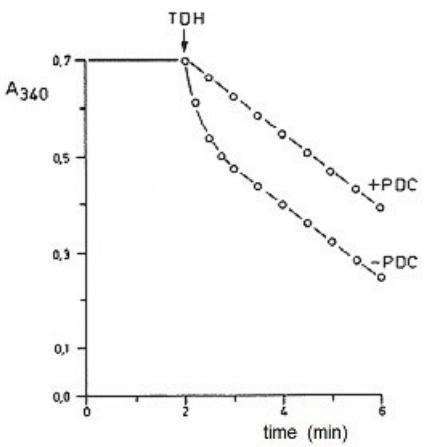
The inability of tropic acid, phenylmalonic acid and phenylacetic acid to serve as a substrate for PDC was also confirmed by manometric experiments. During incubation of these compounds, production of CO_2 was not observed in the presence of PDC.

7.5.2 Specificity for the keto-form of pma

As reported (7.2) decarboxylase has a strong catalytic effect on the decarboxylation of keto-pma. In a similar experiment, enol-pma was incubated in a solution of NADH in presence and absence of PDC during 2 min. Then, TDH was added and the absorption decrease at 340 nm registered (fig 7.7)

Without PDC, the kinetics of the decrease of the absorption observed was as expected. First, a fast initial decrease (phase I) as the result of the hydrogenation of the amount of keto-pma, present at the time of addition of the TDH. Next, a slow decrease (phase II) controlled by the rate of conversion of the remaining enol-pma into the keto-form.

Fig 7.7 Effect of PDC on enol-pma



Curve – PDC: enol-pma (1.5µmol) was incubated with 0.11 mM NADH in 20 mM K-phosphate pH 7.5 at 25^0 during 2 min. Then, 5 U TDH was added.
Curve + PDC: as above but 0.2 U PDC was added on time t=0.

TDH was added to a solution of enol-pma, preincubated with PDC. In this case, the initial fast decrease of the A_{340} was not observed. The decrease of absorption in phase II occurred with exactly the same speed as the in previous experiment. Apparently, the preincubation with PDC results exclusively in the decarboxylation of the keto-pma, whereas enol-pma is not converted by this enzyme.

This shows that PDC is specific for keto-pma. In this experiment, a large amount of TDH and only a small amount of PDC was used. This was in order to prevent that the PDC would affect noticeably the decrease of absorption in phase II by decarboxylation of keto-pma, in this phase formed by the tautomeric rearrangement.

7.5.3 The stability of the enzyme

The stability of the PDC has been investigated at various temperatures using a purified sample, dialyzed against HMP and containing 0.18 mg protein/ml.

The PDC appeared to be much more stable at higher temperature compared with the other tropic acid enzymes. It was not inactivated by an incubation at 65^0 during 30 min; the enzyme was inactivated 50% by incubation at 85^0 30 min.

The stability of PDC during incubation at various pH values has been studied with the same preparation. The enzyme was stable at 25^0 during 30 min in the pH range of 5-10, but was inactivated outside this pH range.

7.5.4 Miscellaneous

The assay of PDC described in this chapter does not allow to establish the pH optimum for this enzyme, because changes in the pH have effect on the TDH and the establishment of the equilibrium as well. But the effect of some additions could be studied that do not affect the tropic acid dehydrogenation in neutral environment. The metal ions: Mg^{++}, Mn^{++} and Ca^{++} (1 mM), EDTA (1 mM), phenylacetaldehyde and phenylacetic acid (1 mM) did not have an effect on the enzymatic decarboxylation.

In the experiments with the technique according to Warburg (7.2), any effect of the cofactors thiamine pyrophosphate and pyridoxal phosphate was not observed either.

Finally, it should be mentioned that the activity of the PDC can be demonstrated titrimetrically as well, at pH 5.0 in a pH-stat. This is possible because the decarboxylation at this pH is accompanied by acid consumption: the CO_2 does not go in solution as bicarbonate. During incubation with pma, acid consumption was observed in this system, which increases after addition of PDC with a factor of 2.5. However, this method is not suitable for an activity assay of the enzyme.

7.6 DISCUSSION

The action of the PDC has been demonstrated in various ways. Production of NADH is accelerated during the enzymatic dehydrogenation of tropic acid in neutral environment after establishment of the equilibrium (see also fig. 6.9 and fig. 6.16). The enzyme stimulates the formation of phenylacetaldehyde and CO_2 and has a direct effect on the stability of keto-pma. These observations lead to the conclusion that it concerns indeed the 2-keto-phenylmalonic semi-aldehyde decarboxylase.

Hydrazine has a similar effect on the equilibrium established in the presence of TDH. Probably this is related as well to the withdrawal of pma from the equilibrium. This could be caused by a reaction of pma with hydrazine to form the hydrazone; however, it is also possible that hydrazine accelerates the decomposition of pma.

Because of the indirect assay of the PDC activity, it is not possible to determine the pH-optimum. The enzyme does not need a cofactor like thiamine pyrophosphate or pyridoxal phosphate. The enzyme has a remarkable resistance against higher temperatures unlike various decarboxylases for other aromatic acids, including the 2,3-dihydroxybenzoic acid decarboxylase (Subba Rao 1967) and the phenylpyruvic acid decarboxylase (Asakawa 1968). These enzymes are very unstable.

Thin layer chromatography has been used to study the substrate specificity of PDC; none of the aromatic acids tested except for pma is converted in the presence of PDC. The PDC does not catalyze the decarboxylation of enol-pma either. It seems probable that PDC is specific for the substrate keto-pma. The specific function of this enzyme in the bacterium is confirmed by studying the induction of PDC to be reported in chapter 10.

Due to the instability of the keto-pma in neutral aqueous environment, it seems on first sight strange that an enzyme with the function to catalyze the decarboxylation of pma is produced in Pseudomonas PMBL-1.

The presence of the decarboxylase could be understood if one assumes that under physiological circumstances - due to the position of the equilibrium catalyzed by TDH – the steady state concentration of the keto-pma is very low. As a consequence, the spontaneous decomposition of pma results in the production of a very low amount of phenylacetaldehyde per unit of time, too low to support the normal growth with tropic acid as sole source of carbon.

The necessity of the presence of the decarboxylase is evident in mutants that have lost exclusively the genetic information for PDC. These are not able any longer to grow with atropine or tropic acid as sole source of carbon (10.7)

In the metabolic pathway of tropic acid, the first step is a NAD^+ dependent dehydrogenation, followed by an enzymatic decarboxylation. From a theoretical point of view, this is very interesting with regard to the use of NAD^+ as cofactor. The position of the equilibrium suggests that the redox potential of NAD^+/NADH is considerably lower as compared with that of tropic acid/pma. NAD^+ as such is therefore not very suitable as hydrogen acceptor for this dehydrogenation. If the TDH would use a hydrogen acceptor with a higher redox-potential, the dehydrogenation as such would proceed easier. However, such a hydrogen acceptor is less favorable for the energy supply of the cell.

Apparently, Pseudomonas PMBL-1 can use NAD^+ as hydrogen acceptor for the dehydrogenation of tropic acid. This might be feasible because in the next irreversible step pma is continuously removed by decarboxylation. This means that the large decrease in free energy as result of the decarboxylation enables the cell to use a hydrogen acceptor of low redox potential in the preceding step.

This is of course very beneficial for the energy supply in the cell.