Prephenate dehydratase of the actinomycete *Amycolatopsis methanolica*: purification and characterization of wild-type and deregulated mutant proteins

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Prephenate dehydratase (PDT) is a key regulatory enzyme in L-phenylalanine biosynthesis in the Gram-positive bacterium *Amycolatopsis methanolica*. The PDT protein was purified to homogeneity (1957-fold) from wild-type cells with a final yield of 6.5%. It was characterized as a 150 kDa homotetrameric protein with a subunit size of 34 kDa. The first 35 N-terminal amino acids were identified, revealing highest similarity to the PDT proteins from *Corynebacterium glutamicum* and *Bacillus subtilis*. Kinetic studies showed that the *A. methanolica* PDT is

allosterically inhibited by phenylalanine and activated by tyrosine. Phenylalanine caused an increase in the $s_{0.5}$ for prephenate and a decrease in the V_{max} . Tyrosine caused a decrease in the $s_{0.5}$ for prephenate and an increase in the V_{max} . Spontaneous *o*-fluoro- and *p*-fluoro-DL-phenylalanine-resistant mutants of *A. methanolica* were isolated. Kinetic studies with the partially purified PDT proteins of strains pFPhe32 and oFPhe84 showed that these mutant proteins had become (partly) insensitive to both phenylalanine inhibition and tyrosine activation.

INTRODUCTION

The aromatic amino acids L-phenylalanine and L-tyrosine are both derived from chorismate, the end product of the shikimate pathway. The last common step in their biosynthesis is catalysed by chorismate mutase, yielding prephenate from chorismate. Phenylalanine biosynthesis further proceeds by conversion of prephenate into phenylpyruvate (via prephenate dehydratase, PDT) or into aroenate (via prephenate aminotransferase). Phenylpyruvate is subsequently transaminated (via aromatic amino acid aminotransferase) and aroenate is dehydrated (via aroenate dehydratase, ADT) to yield phenylalanine in both cases [1].

Depending on the micro-organism studied, the PDT and ADT reactions are catalysed by either a specific PDT or ADT enzyme respectively or a cyclohexadienyl dehydratase (CDT) enzyme, displaying both activities. Both PDT and CDT are present and may coexist in members of the superfamilies A and B of Gram-negative bacteria, providing dual pathways to phenylalanine. In these organisms PDT, but not CDT, is associated with chorismate mutase in a bifunctional protein, the P-protein. Members of the superfamily C of Gram-negative bacteria synthesize phenylalanine via aroenate and possess only a specific ADT enzyme [2]. In contrast, only a specific PDT enzyme has been detected in all Gram-positive bacteria studied so far [3]. The PDT and ADT (but not CDT) enzymes are generally sensitive to feedback regulation by phenylalanine and/or tyrosine [3]. Also metabolically more remote effectors such as tryptophan, methionine and leucine may affect PDT activity [4–7].

The Gram-positive bacterium *Amycolatopsis methanolica* is closely related to *A. mediterranei*, *A. orientalis* and *Saccharopolyspora erythraea*, industrial strains that produce the antibiotics rifamycin, vancomycin and erythromycin respectively [8–10]. In

actinomycetes, rifamycin, vancomycin and many other secondary metabolites are derived from aromatic precursor compounds, intermediates in the shikimate pathway [11,12]. Current knowledge of the biochemistry of the pathways of primary metabolism in these actinomycetes is limited but considered to be important for further rational improvement of strains overproducing aromatic amino acids and derived compounds [13,14].

Here we report the purification and characterization of the PDT enzymes from *A. methanolica* wild-type and two phenylalanine-analogue-resistant mutant strains.

EXPERIMENTAL

Materials

Biochemicals were obtained from Boehringer, Mannheim, Germany. Prephenate was obtained as a barium salt from Sigma. Barium ions were removed via precipitation with excess K_2SO_4 . L-Aroenate was a gift from Professor R. A. Jensen, University of Gainesville, Gainesville, U.S.A. All other chemicals were of analytical grade and commercially available.

Micro-organisms and cultivation

Maintenance of *A. methanolica* wild-type (NCIB 11946), the derived mutants and the plasmid-pMEA300-deficient strain WV2, and the procedures for cultivation in batch cultures, harvesting of cells and growth measurement have all been described previously [15]. Glucose was heat-sterilized (1 M stock solution) and added to the medium at a final concentration of 50 mM.

Abbreviations used: PDT, prephenate dehydratase; ADT, aroenate dehydratase; CDT, cyclohexadienyl dehydratase; oFPhe, *o*-fluoro-DL-phenylalanine; pFPhe, *p*-fluoro-DL-phenylalanine; OPA, *o*-phthalaldehyde.

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Isolation of phenylalanine-analogue-resistant mutants

Mutants of the wild-type strain resistant to the toxic phenylalanine analogues *o*-fluoro-DL-phenylalanine (oFPhe, 27.3 mM) or *p*-fluoro-DL-phenylalanine (pFPhe, 32.8 mM) were isolated on 10 mM glucose mineral agar (1.5 %, w/v) plates containing filter-sterilized analogues. For each analogue, 15 agar plates were inoculated with approx. 5×10^7 cells each. After 2 weeks the spontaneous resistant colonies that had appeared were purified on fresh agar plates with the same composition.

Preparation of extracts and enzyme assays

Washed cell suspensions were disrupted in a French pressure cell at 1000 MPa. Unbroken cells and debris were removed by centrifugation at 40000 *g* for 30 min at 4 °C and the supernatant, containing 10–20 mg of protein/ml, was used for enzyme assays. Unless otherwise stated, enzyme assays were performed at 37 °C.

PDT (EC 4.2.1.51) was assayed by following the formation of phenylpyruvate. The reaction mixture (0.5 ml) contained 1 mM potassium prephenate and 50 mM Tris/HCl, pH 7.5. At appropriate time intervals, 0.5 ml of 2 M NaOH was added and the absorbance of phenylpyruvate was measured at 320 nm [16].

ADT was assayed by measuring the formation of phenylalanine [17].

Purification of PDT

Enzyme purification and the centrifugation steps were performed at 4 °C unless stated otherwise. A System Prep 10 liquid-chromatography system (Pharmacia LKB Biotechnology) was used for all chromatography steps.

Step 1

Glucose-grown cells (25 g wet weight) of *A. methanolicus* strain WV2 were harvested in the exponential growth phase. Cell-free extract was prepared in 25 mM Tris/HCl, pH 7.5, containing 1 mM dithiothreitol (buffer A) as described above. DNAase I and 1 mM MgCl₂ were added to the cell-free extract and incubated for 10 min.

Step 2: Q-Sepharose anion-exchange chromatography

The material was applied to a column (2.6 cm × 30 cm) of Q-Sepharose fast flow at 4 °C equilibrated in buffer A. Bound protein was eluted with a 912 ml increasing linear gradient from 0.15 to 0.40 M NaCl (flow rate 4 ml/min; fractions 12 ml).

Step 3: (NH₄)₂SO₄ precipitation

Solid (NH₄)₂SO₄ was slowly added to the sample to a final concentration of 35 %. The mixture was stirred for 30 min at 4 °C and centrifuged for 15 min at 40000 *g*. The resulting supernatant was adjusted to 50 % by adding solid (NH₄)₂SO₄. The mixture was stirred for 30 min at 4 °C and centrifuged for 15 min at 40000 *g*. The pellet was dissolved in 10 ml of buffer A and dialysed against the same buffer.

Step 4: Mono Q anion-exchange chromatography

Protein from step 3 was divided into three portions. Each portion was applied separately to a Mono Q HR 5/5 anion-exchange column. Bound protein was eluted with a 40 ml linear increasing gradient from 0 to 0.5 M NaCl in buffer A (flow rate 1 ml/min; fractions 1 ml).

Step 5: Hydrophobic interaction chromatography

Protein from step 4 was adjusted to 1.5 M (NH₄)₂SO₄ and applied to an alkyl-Superose HR 5/5 column. Bound protein was eluted with a 25 ml linear decreasing gradient from 1.5 to 0 M (NH₄)₂SO₄ in buffer A (flow rate 0.5 ml/min; fractions 1 ml).

Step 6: Gel-filtration chromatography

Protein from step 5 was concentrated to 0.5 ml with a Microsep ultrafiltration device (cut-off molecular mass, 10 kDa; Filtron, Breda, The Netherlands) and applied to a Superdex 200 HR 16/60 gel-filtration column equilibrated in buffer A (flow rate 1 ml/min; fractions 1 ml).

Step 7: Hydrophobic interaction chromatography

Protein from step 6 was adjusted to 0.7 M (NH₄)₂SO₄ and applied to a phenyl-Superose HR 5/5 column. Bound protein was eluted with a 30 ml linear gradient from 0.7 M (NH₄)₂SO₄ plus 0 % acetonitrile to 0 M (NH₄)₂SO₄ plus 10 % (v/v) acetonitrile in buffer A (flow rate 0.5 ml/min; fractions 0.5 ml). The purified preparations obtained were stored at –20 °C in 40 % (v/v) glycerol.

Partial purification of PDT from mutant strains pFPhe32 and oFPhe84

Step 1

Glucose-grown cells (1 litre; 20 mM glucose) were harvested in the exponential growth phase. Cell-free extract was prepared as described above. DNAase I and 1 mM MgCl₂ were added to the cell-free extract and incubated for 10 min.

Step 2: Mono Q anion-exchange chromatography

Protein from step 1 was divided in three portions and each was applied to a Mono Q HR 5/5 anion-exchange column. Bound protein was eluted with a 30 ml linear increasing gradient from 0 to 0.5 M NaCl in buffer A (flow rate 1 ml/min; fractions 1 ml).

Step 3: (NH₄)₂SO₄ precipitation

Protein from step 2 was adjusted to 55 % (NH₄)₂SO₄. The mixture was stirred for 30 min at 4 °C and centrifuged for 15 min at 40000 *g*. The pellet was dissolved in 1 ml of buffer A.

Step 4: Gel-filtration chromatography

Protein from step 3 was applied to a Superdex 200 HR 16/60 gel-filtration column equilibrated in buffer A (flow rate 1 ml/min; fractions 2 ml).

Step 5: Hydrophobic interaction chromatography

Protein from step 4 was adjusted to 0.8 M (NH₄)₂SO₄ and applied to a phenyl-Superose HR 5/5 column. Bound protein was eluted with a 30 ml linear gradient from 0.8 M (NH₄)₂SO₄ plus 0 % acetonitrile to 0 M (NH₄)₂SO₄ plus 10 % (v/v) acetonitrile in buffer A (flow rate 0.5 ml/min; fractions 1.0 ml).

SDS/PAGE

SDS/PAGE was performed as described by Laemmli and Favre [18] with the following marker proteins from the Combithek

One unit of PDT activity was defined as the amount of enzyme that catalysed the formation of 1 μ mol of phenylpyruvate/min.

	Total activity (units)	Total volume (ml)	Total protein (mg)	Specific activity (units/mg)	Purification (-fold)	Yield (%)
Crude extract	37.2	41	2188	0.017	1	100.0
Q-Sepharose	27.2	19	247	0.11	6	73.1
(NH ₄) ₂ SO ₄ fractionation	19.2	11	73.8	0.26	15	51.6
Mono Q	23.2	10	12.9	1.8	106	62.4
Alkyl-Superose	10.9	2.0	2.0	5.4	318	29.3
Superdex 200	4.7	5.0	0.42	11.1	651	12.6
Phenyl-Superose	2.4	2.0	0.072	33.3	1957	6.5

(a)

Am: SR-----IAYFGPVGTFTHGEAART-----FMAAGDELVAETIPK (1-35)
Bs: MK-----VGYLGPAATFTHLAVSSC-----F-QNGAEHVAYRTYPE (1-35)
Cg: MSDAPTVVAYLGPAGTFTTEALYKFADAGV---FGDGEIEQLPAKSQPE (1-46)
Ps: LR-----VAYLGPEGTFSQAALKH-----FGHSVISK-PMAAIDE (95-129)
Ec: ---SRIATFLGPKGSYSHLAQRQYAAHFHQFIESGCAKFA----D (103-141)

. . . . * *

(b)

Bs: -----MKVGYLGPAATFTTHLAVSSCF-QNG-----AEHVAYRTYPE (1-35)
. . . | . | . | . | . | . | . | . |
Am: -----SRIAYFGPVGTFTHGEAART-FMAAG-----DELVAETIPK (1-35)
. . | . | . | . | . | . | . | . | . |
Ca: MSDAPTVVAYLGPACTFTTEALYK-FADAGVFGDGEIEQLPAKSQPE (1-46)

* or |, identical residues; ., similar residues; -, gap; bold type, identical amino acids in all three sequences.

RESULTS

Purification of PDT

PDT (but not ADT and therefore not CDT) activities were readily detected in *A. methanolica*. To determine the kinetic parameters of PDT the protein was purified from glucose-grown cells harvested when in the exponential growth phase. The purification protocol described reproducibly yielded a homogeneous preparation of PDT, as judged by SDS/PAGE. The final purification was 1957-fold and a final yield of 6.5% was obtained (Table 1).

N-Terminal sequence analysis

The first 35 N-terminal amino acids of the purified PDT were identified (Figure 1). The first amino acid was identified as serine

Figure 2 Kinetics of PDT

(a) Plots of the specific activity of wild-type PDT (v) against various prephenate concentrations in the presence of no effector ($\bullet$), 5 μM phenylalanine ($\blacktriangledown$), 100 μM phenylalanine ($\blacksquare$), 5 μM tyrosine ($\blacktriangle$) and 100 μM tyrosine ($\blacklozenge$); (b) plots of $\log [v/(V_{\text{max}} - v)]$ against $\log$ [prephenate] under the conditions described in (a).

Table 2 Kinetic properties of PDT proteins purified from *A. methanolica* strain WV2 (A), pPhe32 (B) and oPhe84 (C)

Data from Figures 2 and 6 were fitted to the Hill equation:

$$v = V_{\text{max}} \left(\frac{s^h}{s_{0.5}^h + s^h} \right)$$

where v is velocity, s is substrate concentration, V_{max} is maximum velocity, $s_{0.5}$ is substrate concentration at 0.5 V_{max} , and h is the Hill coefficient. The S.D. represents the differences between measured values and data calculated using the Hill equation.

Additions	V_{max} (units/mg)	$s_{0.5}$ (μM)	h
A			
None	38.0 ± 1.9	142 ± 13.9	1.33 ± 0.11
5 μM Phe	21.6 ± 0.7	209 ± 11.9	1.49 ± 0.07
100 μM Phe	13.1 ± 0.9	345 ± 28.1	2.10 ± 0.16
5 μM Tyr	60.5 ± 1.9	96 ± 6.0	1.37 ± 0.10
100 μM Tyr	62.6 ± 2.4	93 ± 7.0	1.49 ± 0.15
B			
None	3.5 ± 0.04	97 ± 2.1	1.43 ± 0.04
100 μM Phe	3.5 ± 0.2	115 ± 11.3	1.19 ± 0.1
1000 μM Phe	3.0 ± 0.1	125 ± 11.8	1.31 ± 0.1
50 μM Tyr	4.4 ± 0.1	97 ± 4.9	1.19 ± 0.06
100 μM Tyr	5.6 ± 0.2	113 ± 7.5	1.11 ± 0.06
C			
None	2.2 ± 0.06	64 ± 4.3	1.12 ± 0.09
100 μM Phe	1.6 ± 0.06	157 ± 11.9	1.30 ± 0.08
1000 μM Phe	1.7 ± 0.08	160 ± 16.0	1.27 ± 0.1
100 μM Tyr	2.1 ± 0.02	44 ± 1.2	1.53 ± 0.07

with a yield of 86 pmol. In cycle 29 a low amount of arginine (12 pmol) was detected together with alanine (30 pmol). Residues 36 and 38 were not identified. Residues 37, 39 and 40 were respectively leucine, arginine and valine (not shown). The *A. methanolica* PDT N-terminal 35 amino acid sequence shared 11.4% (4 of 35) identity with and 37.1% (13 of 35) similarity to those of the PDT proteins from *C. glutamicum* [21], *B. subtilis* [22], and internal parts of the P-proteins from *E. coli* [23] and *Ps. stutzeri* [24] (Figure 1a). Alignment of the *A. methanolica* sequence with those of the *B. subtilis* and *C. glutamicum* PDT proteins revealed 42.9% identical and 60.0% similar residues and 42.9% identical and 57.4% similar residues respectively. The PDT N-terminal amino acid sequences of the three Gram-positive bacteria possessed 28.6% overall identity and 50.0% overall similarity (Figure 1b).

Properties of PDT

SDS/PAGE revealed a single band with a calculated molecular mass of 34 kDa suggesting a tetrameric quaternary structure for the native protein. Gel filtration in the presence of 1 mM phenylalanine or 1 mM tyrosine did not result in a shift in the position of the PDT activity peak (results not shown).

PDT activity was constant in the pH range 6–8.5. A plot of pH against log activity gave pK_a values of 5.5 and 8.5, suggesting that two ionizable groups may be involved in catalysis of prephenate dehydration.

All 20 natural amino acids (1 mM) were tested for their effect on PDT activity (with 200 μM prephenate). In addition to the effects of phenylalanine and tyrosine (see below), methionine activated PDT 1.6-fold and D-phenylalanine caused 48% inhibition. Other amino acids only showed relatively minor effects on PDT activity (less than 15%), if at all.

Figure 3 Effects of phenylalanine (a) and tyrosine (b) on PDT activity

(a) Plots of the relative specific activity (v) of wild-type PDT (○) and the pPhe32 (●) and oPhe84 (■) PDTs against various phenylalanine concentrations in the presence of 200 μM prephenate; (b) plots of the relative specific activity (v) of wild-type PDT (○) and the pPhe32 (●) and oPhe84 (■) PDTs against various tyrosine concentrations in the presence of 200 μM prephenate.

No significant decrease in PDT activity was found during storage at -20°C in a 40% (v/v) glycerol solution for 2 years.

Kinetics of PDT

The relationship between prephenate concentration and activity did not obey Michaelis–Menten kinetics; the data were better fitted to the Hill equation (Figure 2; Table 2). Phenylalanine was a strong inhibitor of PDT activity, causing an increase in the $s_{0.5}$ for prephenate and a decrease in the $V_{\max}$; the Hill coefficient (h) increased with increasing amounts of phenylalanine (Table 2A). At 200 μM prephenate the K_i for phenylalanine was 6.7 μM, and 95% inhibition was calculated at saturating amounts of this amino acid (Figure 3a). Tyrosine strongly activated PDT activity by decreasing the $s_{0.5}$ for prephenate and increasing the $V_{\max}$; the Hill coefficient increased only slightly (Table 2A). The K_a for

Figure 4 Effects of phenylalanine and tyrosine on PDT activity at different prephenate concentrations

Plots of wild-type PDT relative specific activity (v) in the presence of 100 μM phenylalanine (●, left axis) or 100 μM tyrosine (○, right axis) against various prephenate concentrations are shown; 100% values correspond to $V_{\max}$ in the absence of effectors.

Figure 5 Plots of wild-type PDT specific activity against various tyrosine concentrations in the presence of 200 μM prephenate and 0.5 mM (●) or 2.5 mM (○) phenylalanine

tyrosine was 4.1 μM, and 258% activation was calculated at saturating amounts of the amino acid (Figure 3b). The effects of tyrosine activation and phenylalanine inhibition were greatest at low prephenate concentrations (Figure 4). In the presence of phenylalanine, PDT activity could still be activated by tyrosine. At relatively low phenylalanine concentrations (0.5 mM), tyrosine was able to produce its normal extent of activation, but at 2.5 mM phenylalanine the tyrosine-activating effect was less strong (Figure 5).

Deregulated PDT mutants

De Boer et al. [25] provided evidence that the toxic phenylalanine analogues pPhe and oPhe block growth of *A. methanolica* via

Figure 6 Effects of phenylalanine and tyrosine on the activity of the PDT mutants pFphe32 (a) and oFphe84 (b)

(a) Plots of PDT^{pFphe32} specific activity (v) against various prephenate concentrations in the presence of no effector (●), 100 μM phenylalanine (■), 1000 μM phenylalanine (□), 50 μM tyrosine (▲) and 100 μM tyrosine (◆); (b) plots of PDT^{oFphe84} specific activity (v) against various prephenate concentrations in the presence of no effector (●), 100 μM phenylalanine (■), 1000 μM phenylalanine (□) and 100 μM tyrosine (◆).

inhibition of PDT. In the present study we observed that pFphe- and oFphe-resistant mutant strains of wild type *A. methanolicus* could be readily isolated. After 2 weeks of incubation, numerous spontaneous resistant colonies were clearly visible against a background of tiny colonies. Initially, a total of 400 colonies resistant to either one of the analogues was selected. After repeated transfers, 75 pFphe- and 122 oFphe-resistant mutants clearly still scored positive. The PDT enzymes in crude extracts of five pFphe- and five oFphe-resistant strains were analysed for their sensitivity to feedback regulation. Strains pFphe32 and oFphe84 clearly possessed mutant PDT enzymes; the other strains displayed wild-type feedback regulation for PDT.

PDT activities in crude extracts of strains pFphe32 and oFphe84 ranged from 15 to 20 m-units/mg of protein, comparable with PDT activity in the wild-type (Table 1). The PDT proteins of strains pFphe32 (PDT^{pFphe32}; 233-fold purification, 24% yield) and oFphe84 (PDT^{oFphe84}; 110-fold purification,

22% yield) were partially purified. During the purification both proteins behaved essentially the same as the wild-type (PDT^{wt}). The molecular masses of both mutant PDT proteins were estimated to be 150 kDa.

The effects of phenylalanine and tyrosine on the $s_{0.5}$ of PDT^{pFphe32} for prephenate were smaller than on the $s_{0.5}$ of PDT^{wt}. In the presence of 100 and 1000 μM phenylalanine the V_{max} decreased only slightly. An increase in V_{max} was observed in the presence of tyrosine (Figure 6a, Table 2B). At high phenylalanine concentrations its inhibitory effect was lost and a small activation (to a maximum of 125%) of PDT activity even occurred (Figure 3a). At 200 μM prephenate the K_s of tyrosine activation was 184 μM and 249% activation was calculated at saturating amounts of the amino acid (Figure 3b).

The $s_{0.5}$ of PDT^{oFphe84} for prephenate was lower than those of the PDT^{wt} and PDT^{pFphe32} proteins. In the presence of tyrosine the $s_{0.5}$ constant of PDT^{oFphe84} became even lower. Phenylalanine caused an increase in the $s_{0.5}$ of PDT^{oFphe84} for prephenate whereas the V_{max} decreased (Figure 6b, Table 2c). PDT enzyme activity was less sensitive to feedback inhibition at high concentrations of phenylalanine. At 200 μM prephenate, the K_i for phenylalanine was 9.1 μM , but only 47% inhibition was calculated at saturating amounts of the amino acid (Figure 3a). Tyrosine activation had decreased considerably; at 200 μM prephenate the K_s of tyrosine activation was 29 μM , and 126% activation was calculated at saturating amounts of this amino acid (Figure 3b).

DISCUSSION

Many actinomycetes are important in the industrial production of primary and secondary metabolites, including aromatic amino acids and the various antibiotics derived from them [12]. Detailed studies of the regulation of the biosynthesis of aromatic amino acids have not been carried out in actinomycetes. We are currently engaged in an analysis of the enzymology and regulation of the shikimate pathway in the actinomycete *A. methanolicus*. As a first approach, several of the enzymes involved have been purified and kinetically characterized [10] (this study). The present paper is the first report of the purification to homogeneity and detailed characterization of a monofunctional tyrosine-activated PDT from a bacterial source (Table 1).

A single PDT enzyme was detected in crude extracts of *A. methanolicus*; ADT and CDT activities were not observed. Mutants blocked in PDT are phenylalanine auxotrophs (G. J. W. Euverink, unpublished work). These data show that *A. methanolicus* employs only one pathway for phenylalanine biosynthesis, and that involves PDT. *Pseudomonas aeruginosa* and other Gram-negative bacteria possess a CDT enzyme which is located in the periplasmic space. The physiological role of this widely distributed enzyme remains unknown [26]. Gram-positive bacteria do not possess a periplasmic space, which may explain why CDT enzymes are not detected in these organisms. PDT activity was not associated with chorismate mutase activity in *A. methanolicus*, as also found in other Gram-positive bacteria, such as *B. subtilis* [5], *Brevibacterium flavum* [27,28], *C. glutamicum* [29] and *Streptomyces aureofaciens* [30]. Thus far, bifunctional chorismate mutase-PDT proteins have been found in Gram-negative bacteria only [2].

Characterization of the purified *A. methanolicus* PDT enzyme revealed that, in view of its subunit size (34 kDa) and N-terminal amino acid sequence (Figure 1), it is clearly related to PDT enzymes from other sources, especially the Gram-positive bacteria *C. glutamicum* and *B. subtilis*. Clear differences were

observed as well, however. *A. methanolica* PDT is a 150 kDa homotetramer, allosterically inhibited by phenylalanine and activated by tyrosine (Figure 2). The *C. glutamicum* and *Br. flavum* PDT enzymes have been studied in crude extracts or partially purified PDT preparations only. These enzymes are larger (220–260 kDa), probably octamers with 33 kDa subunits (deduced from the DNA sequence) and inhibited not only by phenylalanine but also by tryptophan, and activated by tyrosine [6,7,21]. The molecular mass of the *A. methanolica* PDT protein was not affected by phenylalanine or tyrosine. This indicated that regulation of PDT activity in *A. methanolica* does not involve the dissociation/association phenomena observed for the *B. subtilis* and *E. coli* PDT enzymes [31,32]. Studies on partially purified enzymes from various other actinomycetes suggested that the PDT enzymes from Actinoplanaceae [33] and *Nocardia mediterranei* U-32 [34] are also inhibited by phenylalanine and activated by tyrosine; in representatives of the Micromonosporaceae, Nocardiaceae and Streptomyetaceae families, PDT activity was inhibited by phenylalanine and inhibited or activated by tyrosine [33,35,36]. The remote effector methionine clearly inhibited PDT activity in *A. methanolica*. Other remote effectors of the PDT enzyme in *B. subtilis* [5,31] and *C. glutamicum* [4,7] (tryptophan, leucine, isoleucine and histidine) did not affect *A. methanolica* PDT.

Phenylalanine analogues have been employed in the isolation of mutants with deregulated PDT enzymes in various organisms. Analysis of *m*-fluoro-DL-phenylalanine-resistant mutants of *C. glutamicum* revealed phenylalanine-feedback-inhibition-resistant PDT enzymes with wild-type (1), or derepressed enzyme synthesis (2), with wild-type regulation but a decreased K_m for prephenate (3) or constitutive enzyme synthesis (4) [7]. Two types of mutant PDT proteins have been isolated from *B. subtilis*, using toxic β -2-thienylalanine: a hyperactive PDT that had become insensitive to phenylalanine, tryptophan, methionine and leucine inhibition (1), and a PDT with wild-type activity that was not activated by phenylalanine, tryptophan, methionine and leucine (2) [5].

The analogues oPhe and pPhe were successfully used for the isolation of *A. methanolica* PDT mutants with altered regulatory properties (Figures 3 and 6, Table 2). The mutant PDT protein characterized from strain pPhe32 had become (almost) completely insensitive towards phenylalanine feedback inhibition. PDT^{oPhe84} and PDT^{wt} had similar K_i values for phenylalanine but maximal phenylalanine inhibition was only 47% for PDT^{oPhe84}. Both mutant PDT enzymes were still activated by tyrosine, albeit less effectively than PDT^{wt}. Maximal activation at saturating tyrosine concentrations was considerably lower with PDT^{oPhe84}. Compared with PDT^{wt} both mutant enzymes displayed a decreased $s_{0.5}$ for prephenate (in the absence of tyrosine). The $s_{0.5}$ of PDT^{oPhe84} (but not PDT^{oPhe32}) for prephenate decreased even further in the presence of tyrosine (Table 2). The PDT specific activities in crude extracts of both mutants indicated that these enzymes possessed V_{max} values comparable with that of the wild type. These types of PDT mutant enzyme have not been described before. In *E. coli* and *Erwinia herbicola*, point mutations or deletions in the C-termini of the respective *pheA* genes were found to result in feedback-inhibition-resistant PDT enzymes [37,38]. The cloning and sequencing of genes encoding the different *A. methanolica* PDT (mutant) enzymes in future work is expected to provide further insight into the sites involved in phenylalanine inhibition and tyrosine activation.

The kinetic data presented show that PDT^{wt} of *A. methanolica* is a key regulatory enzyme. The organism uses single pathways for phenylalanine and tyrosine biosynthesis from prephenate, and none of the other enzymes involved appear to be regulated

at the activity level [39] (this study). In wild-type *A. methanolica*, the relative contribution of prephenate to phenylalanine and tyrosine biosynthesis is therefore probably controlled by modification of the $s_{0.5}$ of PDT for prephenate and its V_{max} , depending on the intracellular concentrations of the amino acids (Figure 5). Recruitment of the mutant proteins PDT^{pPhe32} and PDT^{oPhe84}, which are (almost) completely insensitive to phenylalanine inhibition and display a low $s_{0.5}$ for prephenate, resulted in minor accumulation of phenylalanine by the mutant strains only. This may be explained by the presence of further steps in aromatic amino acid metabolism, at 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase and chorismate mutase, which are sensitive to phenylalanine feedback inhibition [39] or by phenylalanine catabolism [15]. The PDT mutant strains displayed normal growth behaviour, showing that they are still able to synthesize sufficient tyrosine. The specific activity of prephenate aminotransferase *in vivo* and its affinity for prephenate therefore must be high enough in *A. methanolica* to compete with these deregulated mutant PDT enzymes with low $s_{0.5}$ values for prephenate.

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REFERENCES

- Garner, C. and Herrmann, K. M. (1983) in *Amino Acids: Biosynthesis and Genetic Regulation* (Herrmann, K. M. and Somerville, R. L., eds.), pp. 323–338, Addison-Wesley, London
- Jensen, R. A. and Ahmad, S. (1991) *Trends Ecol. Evol.* **5**, 219–223
- Bentley, R. (1990) *Crit. Rev. Biochem. Mol. Biol.* **25**, 307–384
- Hagino, H. and Nakayama, K. (1974) *Agric. Biol. Chem.* **38**, 2367–2376
- Pierson, D. L. and Jensen, R. A. (1974) *J. Mol. Biol.* **90**, 563–579
- Sugimoto, S. and Shilo, I. (1974) *J. Biochem. (Tokyo)* **76**, 1103–1111
- Fazel, A. M. and Jensen, R. A. (1980) *Arch. Biochem. Biophys.* **200**, 165–176
- De Boer, L., Dijkhuizen, L., Grobbee, G. et al. (1990) *Int. J. Syst. Bacteriol.* **40**, 194–204
- Embley, T. M. (1991) in *The Prokaryotes* (Balows, A., Trüper, H. G., Dworkin, M., Harder, W. and Schleifer, K.-H., eds.), pp. 996–1027, Springer Verlag, New York
- Euverink, G. J. W., Hessels, G. I., Vrijbloed, J. W., Coggins, J. R. and Dijkhuizen, L. (1992) *J. Gen. Microbiol.* **138**, 2449–2457
- Gygax, D., Ghisalba, O., Treichler, H. and Nüesch, J. (1990) *J. Antibiot.* **43**, 324–326
- Haslam, E. (1993) *Shikimic Acid: Metabolism and Metabolites*, John Wiley & Sons Ltd, Chichester
- Bushell, M. E. (1989) in *Microbial Products: New Approaches* (Baumberg, S., Hunter, I. S. and Rhodes, P. M., eds.), pp. 95–120, Cambridge University Press, Cambridge
- Kell, D. B., Van Dam, K. and Westerhoff, H. V. (1989) in *Microbial Products: New Approaches* (Baumberg, S., Hunter, I. S. and Rhodes, P. M., eds.), pp. 61–93, Cambridge University Press, Cambridge
- De Boer, L., Harder, W. and Dijkhuizen, L. (1988) *Arch. Microbiol.* **149**, 459–465
- Patel, N., Pierson, D. L. and Jensen, R. A. (1977) *J. Biol. Chem.* **252**, 5839–5846
- Fischer, R. and Jensen, R. A. (1987) *Methods Enzymol.* **142**, 495–502
- Laemmli, U. K. and Favre, K. (1973) *J. Mol. Biol.* **80**, 575–599
- Higgins, D., Fuchs, R. and Bleasby, A. J. (1992) *Comput. Appl. Biosci.* **8**, 189–191
- Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–254
- Follettie, M. T. and Sinskey, A. J. (1986) *J. Bacteriol.* **167**, 695–702
- Trach, K. and Hoch J. A. (1989) *J. Bacteriol.* **171**, 1362–1371
- Hudson, G. S. and Davidson, B. E. (1984) *J. Mol. Biol.* **180**, 1023–1051
- Fischer, R. S., Zhao, G. S. and Jensen, R. A. (1991) *J. Gen. Microbiol.* **137**, 1293–1301
- De Boer, L., Grobbee, G., Vrijbloed, J. W. and Dijkhuizen, L. (1990) *Appl. Microbiol. Biotechnol.* **33**, 183–189
- Zhao, G. S., Xia, T. H., Aldrich, H. and Jensen, R. A. (1993) *J. Gen. Microbiol.* **139**, 807–813
- Shilo, I. and Sugimoto, S. (1979) *J. Bacteriol.* **86**, 17–25
- Sugimoto, S. and Shilo, I. (1980) *J. Bacteriol.* **88**, 167–176

- 29 Hagino, H. and Nakayama, K. (1975) *Agric. Biol. Chem.* **39**, 331–342
- 30 Görisch, H. (1987) *Methods Enzymol.* **142**, 464–472
- 31 Riepl, R. G. and Glover, G. I. (1979) *J. Biol. Chem.* **254**, 10321–10328
- 32 Baldwin, G. S., McKenzie, G. H. and Davidson, B. E. (1981) *Arch. Biochem. Biophys.* **211**, 76–85
- 33 Hund, H. K., Keller, B. and Lingens, F. (1987) *Z. Naturforsch.* **42c**, 387–393
- 34 Xia, T. H. and Chiao, J. S. (1989) *Biochim. Biophys. Acta* **991**, 6–11
- 35 Lowe, D. A. and Westlake, D. W. S. (1972) *Can. J. Biochem.* **50**, 1064–1073
- 36 Speth, A. R., Hund, H. K. and Lingens, F. (1989) *Biol. Chem. Hoppe-Seyler* **370**, 591–599
- 37 Nelms, J., Edwards, R. M., Warwick, J. and Fotheringham, I. (1992) *Appl. Environ. Microbiol.* **58**, 2592–2598
- 38 Xia, T. H., Zhao, G. S. and Jensen, R. A. (1992) *Appl. Environ. Microbiol.* **58**, 2792–2798
- 39 De Boer, L., Vrijbloed, J. W., Grobbee, G. and Dijkhuizen, L. (1989) *Arch. Microbiol.* **151**, 319–325

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