The 8-amino-7-oxopelargonate synthase from *Bacillus sphaericus*

Purification and preliminary characterization of the cloned enzyme overproduced in *Escherichia coli*

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The 8-amino-7-oxopelargonate synthase [6-carboxyhexanoyl-CoA:L-alanine carboxyhexanoyltransferase (decarboxylating); EC 2.3.1.47] from *Bacillus sphaericus* involved in biotin biosynthesis was purified from an *Escherichia coli* overproducing strain. The purification afforded an electrophoretically homogeneous enzyme with a specific activity of 0.67 unit/mg. The purified enzyme is a monomer of 41 kDa. N-Terminal sequencing of the first 14 amino acid residues showed complete agreement with the predicted sequence from the *bioF* gene. The pure enzyme showed the characteristic absorption band (425 nm) of pyridoxal 5'-phosphate-dependent enzymes. Furthermore, the holoenzyme was resolved during an affinity step yielding the inactive apoenzyme, which recovered activity and the 425 nm-absorption band on dialysis against pyridoxal 5'-phosphate. K_m values for L-alanine and pimeloyl-CoA were respectively 3 mM and 1 μ M.

INTRODUCTION

As part of a general project on the biosynthesis of biotin, i.e. fundamental aspects (enzymology, genetics) as well as industrial microbiological production of biotin, we have investigated the second step of this metabolic pathway in *Bacillus sphaericus*: the transformation of pimeloyl-CoA into 8-amino-7-oxopelargonate (AOP) (Scheme 1) [1,2]. This interesting reaction is catalysed by AOP synthase [6-carboxyhexanoyl-CoA:L-alanine carboxyhexanoyltransferase (decarboxylating); EC 2.3.1.47], which has been detected in the cell-free extract of different bacteria [3,4], and partially purified from *B. sphaericus* by Izumi and colleagues [5]. This enzyme belongs to the family of pyridoxal 5'-phosphate-dependent enzymes that catalyse the condensation of an acyl-CoA with an amino acid leading to an α -amino ketone [5-aminolaevulinate synthase (EC 2.3.1.37), 2-amino-3-oxobutyrate:CoA ligase (EC 2.3.1.29) and serine palmitoyltransferase (EC 2.3.1.50)]. However, our knowledge of the mechanism of these enzymes is restricted to the pioneering work of Jordan and co-workers on 5-aminolaevulinate synthase [6]. Thus elucidation of the catalytic mechanism of the AOP synthase would be of value and interest and could contribute to the elaboration of specific inhibitors as potential antimicrobial agents. We report here the purification to homogeneity as well as initial characterization of the cloned AOP synthase from *B. sphaericus*, overproduced in *Escherichia coli*.

EXPERIMENTAL

Materials

Phenyl-Sepharose CL-4B, DEAE-Sepharose CL-6B and Red active Red 120-agarose 3000-CL were purchased from Sigma

Chemical Co. (St. Louis, MO, U.S.A.). Sephadex G-100 was from Pharmacia (Uppsala, Sweden). BSA (fraction V) was from Sigma Chemical Co. All microbiological reagents were from Difco Laboratories (Detroit, MI, U.S.A.). All chemicals were of the highest purity available and were purchased from Sigma Chemical Co., Prolabo (Paris, France) or Aldrich Chemical Co. (Milwaukee, WI, U.S.A.).

Methods

H.p.l.c. was performed on a Waters (Milford, MA, U.S.A.) system and f.p.l.c. on a Pharmacia system. N.m.r. spectra were recorded on a JEOL (Tokyo, Japan) FTX 90Q spectrometer. U.v.-visible measurements were recorded on a Kontron (Rotkreuz, Switzerland) Uvikon-930 spectrometer. Electrophoresis was performed on a Bio-Rad Laboratories (Richmond, CA, U.S.A.) apparatus. Sonication was performed on an M.S.E. (Crawley, Sussex, U.K.) 150 W ultrasonic disintegrator. Centrifugations were in a Du Pont (Newton, CT, U.S.A.) Sorval RC5 centrifuge. Elementary analyses were performed at the Service Central de Microanalyse de l'Université Pierre et Marie Curie.

Syntheses

6-(Phenylthiocarbonyl)hexanoic acid (I). Pimelic acid (1.5 g, 9.4 mmol) was treated with an excess of freshly distilled thionyl chloride (2 ml) at 60 °C for 1 h. After evaporation under vacuum, the resultant oily material was dissolved in methylene dichloride (2 ml), and thiophenol (1 ml, 9.1 mmol) was added. The mixture was stirred at 30 °C for 1 h. The organic solution was then washed three times with aq. 2% (w/v) NaHCO_3 . The aqueous phases were combined, acidified with conc. HCl to pH 1 and extracted with methylene dichloride. The organic phases were combined, dried over Na_2SO_4 and evaporated. The mono-

Scheme 1. Reaction catalysed by AOP synthase

Abbreviations used: AOP, 8-amino-7-oxopelargonate; AOP-synthase, 8-amino-7-oxopelargonate synthase [6-carboxyhexanoyl-CoA:L-alanine carboxyhexanoyltransferase (decarboxylating); EC 2.3.1.47].

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thioester, dithioester and remaining pimelic acid were separated by silica-gel chromatography (cyclohexane/ethyl acetate/acetic acid; 15:4:1, by vol.). This procedure yielded compound (I) (900 mg; 37% yield) as crystals, m.p. 45–46 °C. ^1H n.m.r. δ (p.p.m.) (^3H)chloroform, tetramethylsilane) 1.2–1.9 [6H, m, (CH_2)₃], 2.3 (2H, m, CH_2CO_2), 2.6 (2H, m, CH_2COS), 7.4 (5H, s, C_6H_5), 10.6 (1H, s, CO_2H). (Found: C, 61.72; H, 6.44. Calc. for $\text{C}_{13}\text{H}_{16}\text{O}_5\text{S}$: C, 61.88; H, 6.39%.)

Pimeloyl-CoA (II). The transesterification was performed as described by Wieland & Rueff [7] for the synthesis of acyl-CoA thiol esters. CoA (lithium salt), (50 mg, 65 μmol) was dissolved in 1 ml of degassed 2% (w/v) NaHCO_3 , and compound (I) (35 mg, 130 μmol) dissolved in 0.5 ml of methanol was added. The pH was adjusted to 8 by addition of 2% (w/v) NaHCO_3 and the solution was stirred at room temperature. After the reaction was completed (analysed by h.p.l.c.) the pH was adjusted to 2 by the addition of conc. HCl. The aqueous phase was washed three times with diethyl ether and concentrated under reduced pressure. Pimeloyl-CoA was purified by h.p.l.c. on a LiChrospher 100 RP-8e 5 μm (Merck) column with 15% (v/v) acetonitrile in 0.25 M-triethylammonium phosphate buffer, pH 5.5, as eluent (2 ml/min). Compound (II) was kept in an acidic solution (pH 2) at -20°C without noticeable degradation.

6-(Hydroxamylcarbonyl)hexanoic acid (III). Compound (I) (200 mg, 0.8 mmol) was treated with 0.5 ml of 4 M-hydroxylamine in methanol, freshly prepared by mixing 1 vol. of 4 M-hydroxylamine hydrochloride in methanol and 1 vol. of 3.5 M-NaOH in methanol (KCl was eliminated by filtration). The hydroxamic acid was quantitatively formed and was purified by silica-gel chromatography (chloroform/methanol/acetic acid, 14:4:1, by vol.) followed by anion-exchange chromatography (Dowex 1-X4, formate, eluted with a linear formic acid gradient of 0.2–1 M); 100 mg (70% yield) of crystals was obtained, m.p. 120 °C. ^1H n.m.r. δ (p.p.m.) ($^2\text{H}_2\text{O}$, 2-methylpropan-2-ol) 1.2–1.8 [6H, m, (CH_2)₃], 2.0–2.4 (4H, m, CH_2CO). (Found: C, 48.17; H, 7.46; N, 7.80. Calculated for $\text{C}_7\text{H}_{13}\text{NO}_4$: C, 47.99; H, 7.48; N, 7.99%.)

Assays

Pimeloyl-CoA was determined colorimetrically as its hydroxamate [8]. The sample was dissolved in 100 mM-sodium acetate buffer, pH 5.5 (final volume 400 μl), then 200 μl of 4 M-hydroxylamine was added. The mixture was kept at room temperature for 5 min, and then 200 μl of 3 M-HCl and 200 μl of 3% (w/v) FeCl_3 in 0.1 M-HCl were successively added. The absorbance was measured at 500 nm against an appropriate blank. Pure compound (III) gave: ϵ_{500} 761 $\text{M}^{-1}\text{cm}^{-1}$.

AOP synthase was determined by the method originally described by Izumi *et al.* [5] with modifications. An Eppendorf tube containing 7.5 nmol of pimeloyl-CoA, 2.5 μmol of L-alanine, 5 nmol of pyridoxal 5'-phosphate and 2.5 μmol of potassium phosphate buffer, pH 7.0, in a volume of 45 μl was warmed to 37 °C. Then the enzyme was added (5 μl) and the solution was rapidly vortex-mixed before incubation. The reaction was quenched at various times (15 s to 5 min) by the addition of trichloroacetic acid [3% (w/v) final concentration]. After centrifugation, if necessary, 5 μl of the supernatant was used for the agar diffusion bioassay of AOP. Briefly, a 2% (w/v) agar plate (24 cm $\times$ 24 cm) was prepared according to the method of Snell *et al.* [9], autoclaved (80 kPa), for 15 min, cooled and inoculated with *Saccharomyces cerevisiae* A.T.C.C. 9698 (freshly harvested washed cells). After drying for 2 h, 5 μl of standard solutions of AOP (from 0.5 μM to 20 μM) and 5 μl of unknowns were pipetted on to paper discs (6 mm) placed on the agar surface. The plate was then incubated overnight at 30 °C. Plots of growing diameters versus the logarithm of AOP concentration gave a good linear

correlation. One unit of AOP synthase is defined as 1 μmol of AOP produced/min in the conditions described above.

Protein assay

Protein concentrations were determined by the method of Bradford [10] with the reagent supplied by Bio-Rad Laboratories. BSA was used as a standard.

Culture

B. sphaericus AOP synthase was purified from an *E. coli* overproducing strain C268 (Sm^R , ΔbioA , *his*) [11], containing the synthase cloned gene (*bioF*) downstream of a *lac* promoter inserted into plasmid pTG3401 (R. Gloeckler & Y. Lemoine, unpublished work). *E. coli* C268/pTG3401 was grown to early stationary phase at 37 °C in Luria broth medium (200 ml batches in Erlenmeyer flasks), supplemented with 100 μg of ampicillin/ml, and collected by centrifugation (4000 *g* for 15 min). The cells were washed with 50 mM-potassium phosphate buffer, pH 7.0, containing 50 mM- MgSO_4 and 0.4 M-NaCl, centrifuged and kept at -20°C until use.

Enzyme purification

All manipulations were carried out at 4 °C.

Crude extract. The cells were thawed on ice and suspended in buffer A [20 mM-potassium phosphate buffer, pH 7.0, containing 5 mM-2-mercaptoethanol, 20% (v/v) glycerol, 100 μM -pyridoxal 5'-phosphate, 2 mM-EDTA, 1 mM-phenylmethanesulphonyl fluoride and 1 μM -pepstatin A] and disrupted by sonication for 5 min (five 1 min pulses with intervening 1 min cooling periods). The cellular debris was removed by centrifugation (27000 *g* for 20 min).

Clarification. The supernatant was made 1.4 M in $(\text{NH}_4)_2\text{SO}_4$ by the slow addition of crystals, and stirred for 30 min. After centrifugation (27000 *g*, 15 min), the supernatant was made 3 M in NaCl by the addition of crystals.

Phenyl-Sepharose. The supernatant was loaded on a phenyl-Sepharose column (2.5 cm $\times$ 18 cm) equilibrated with buffer B [20 mM-potassium phosphate buffer, pH 7.0, containing 5 mM-2-mercaptoethanol, 20% (v/v) glycerol, 100 μM -pyridoxal 5'-phosphate, 0.5 mM-EDTA and 3 M-NaCl]. After the column had been washed with 100 ml of buffer B, the proteins were eluted with a linear gradient (3 M- to 0 M-NaCl in buffer B, 2 $\times$ 200 ml). Active fractions were pooled, concentrated by ultrafiltration (Amicon P 10) and dialysed against buffer C [20 mM-potassium phosphate buffer, pH 7.3, containing 5 mM-2-mercaptoethanol, 20% (v/v) glycerol, 100 μM -pyridoxal 5'-phosphate and 0.5 mM-EDTA].

DEAE-Sepharose. The enzyme solution was loaded on to a DEAE-Sepharose column (1.5 cm $\times$ 40 cm) equilibrated with buffer C. After the column had been washed with 100 ml of buffer C, the proteins were eluted with a linear salt gradient (0 M- to 0.25 M-NaCl in buffer C, 2 $\times$ 200 ml). Active fractions were pooled, concentrated by ultrafiltration (Amicon P 10) and dialysed against buffer D [20 mM-potassium phosphate buffer, pH 7.0, containing 5 mM-2-mercaptoethanol, 20% (v/v) glycerol and 0.5 mM-EDTA].

Reactive Red-Agarose. The enzyme solution was loaded on to a Reactive Red-Agarose column (2.5 cm $\times$ 13 cm) equilibrated with buffer D. After the column had been washed with 100 ml of buffer D, the proteins were eluted with a linear salt gradient (0 M- to 1.7 M-NaCl in buffer D, 2 $\times$ 200 ml). Active fractions were pooled, concentrated by ultrafiltration (Amicon P 10) and dialysed against buffer E (10 mM-Tris/HCl buffer, pH 7.5, containing 5 mM-2-mercaptoethanol, 0.5 M-EDTA and 100 μM -pyridoxal 5'-phosphate).

F.p.l.c. The enzyme was finally purified on a Mono Q HR 5/5

f.p.l.c. column with a biphasic linear salt gradient (0 M- to 150 mM-NaCl in 10 mM-Tris/HCl buffer, pH 7.5). Active fractions were pooled, concentrated (Amicon Centriprep 10) and dialysed against 20 mM-potassium phosphate buffer, pH 7.0, containing 5 mM-2-mercaptoethanol, 50 % (v/v) glycerol, 100 μ M-pyridoxal 5'-phosphate, 0.1 mM-EDTA and 100 mM-NaCl. The enzyme was kept at 0 °C.

Molecular-mass determination

The subunit molecular mass was determined by SDS/PAGE [12] with the following standards: BSA, 66 kDa; ovalbumin, 45 kDa; glyceraldehyde-3-phosphate dehydrogenase, 36 kDa; carbonic anhydrase, 29 kDa; trypsinogen, 24 kDa; trypsin inhibitor, 20.1 kDa; α -lactalbumin, 14.2 kDa. The proteins were stained with Coomassie Brilliant Blue R-250. The native molecular mass was determined by gel-permeation chromatography on a Sephadex G-100 column (1.6 cm $\times$ 80 cm) with 20 mM-potassium phosphate buffer, pH 7.0, as solvent. The molecular-mass standards utilized were the following: Blue Dextran, void volume; alcohol dehydrogenase, 150 kDa; BSA, 66 kDa; carbonic anhydrase, 29 kDa; cytochrome c, 12.4 kDa; aprotinin, 6.5 kDa.

Amino acid analysis and N-terminal sequence

N-Terminal amino acid sequence analysis was performed by automated Edman degradation in a 477A microsequencer equipped with an on-line phenylthiohydantoin h.p.l.c. analyser and a microcomputer (Applied Biosystems, Foster City, CA, U.S.A.). Amino acid analysis was performed on a 420A amino acid analyser equipped with an on-line h.p.l.c. analyser and a microcomputer (Applied Biosystems), after hydrolysis with 6 M-HCl (Pico-Tag; Millipore-Waters).

Isoelectrofocusing

The isoelectric point was determined on a polyacrylamide gel with either a 3–10 or a 5–7 pH gradient (BioLyte from Bio-Rad Laboratories). The bands were stained with Crocein Scarlet 7B and Coomassie Brilliant Blue R-250. Standards were from Bio-Rad Laboratories.

RESULTS AND DISCUSSION

Syntheses and assays

Pimeloyl-CoA was synthesized as described by Eisenberg & Star [3] starting from pimeloyl chloride rather than from the polymeric anhydride of pimelic acid, the former being readily available, in contrast with the latter. Pimeloyl-CoA was routinely determined as its hydroxamate [8]. Standard calibrations were obtained with the synthetic hydroxamate of pimelic acid (ϵ_{500} 761 M⁻¹·cm⁻¹, linear up to 1 mM). The synthesis of racemic AOP has already been described [13], but we obtained this

compound via a different route (O. Ploux & A. Marquet, unpublished work). For the determination of the AOP synthase activity we have modified the assay originally described by Izumi *et al.* [5]. The assay is based on the determination of the AOP formed over a 5 min period of time (rather than hours) in order to avoid enzyme inactivation. AOP was determined by using the *S. cerevisiae* microbiological agar diffusion assay. Different values for the bioactivity of AOP (in the yeast assay) have been reported in the literature [14,15], but we found that racemic AOP was as active as biotin in the concentration range we used (0.1–20 μ M), in accordance with Eisenberg & Maseda [14]. This routine discontinuous assay for AOP synthase is quite sensitive, but the manual measurement of growth diameters limits its accuracy (5–10 %).

Purification

B. sphaericus AOP synthase was purified from an *E. coli* overproducing strain. Overproduction led to about 1 % of the soluble proteins in *E. coli* as the desired enzyme (calculated from the specific activities). Table 1 summarizes the four-step chromatographic procedure, which produced a 100-fold purification of the enzyme; 2 mg of pure enzyme was obtained starting from 11 g of *E. coli* cell paste. The specific activity obtained, 0.67 unit/mg of protein, is 3000 times higher than the specific activity reported by Izumi *et al.* [5] (0.2×10^{-3} unit/mg), confirming the low purity of their preparation.

Proteinase inhibitors (phenylmethanesulphonyl fluoride, pepstatin A and EDTA) were only useful during the first stage of the purification. Although Izumi *et al.* [5] reported that 1 mM-diisopropyl phosphorofluoridate inhibited the AOP synthase, we

Fig. 1. SDS/PAGE analysis of the enzyme preparation during purification

Lane 1, crude extract; 2, after Phenyl-Sepharose chromatography; 3, after DEAE-Sepharose chromatography; 4, after Reactive Red-agarose chromatography; 5, after f.p.l.c. (Mono Q); 6, molecular-mass markers.

Table 1. Purification of AOP synthase starting from 11 g (wet wt.) of *E. coli* cells

Step	Volume (ml)	Protein (mg)	Activity (units)	Specific activity (munits/mg of protein)	Yield (%)	Purification (fold)
Crude extract	62	1300	7.80	6	—	—
(NH ₄) ₂ SO ₄	62	690	5.50	8	70	1.2
Phenyl-Sepharose	66	139	5.50	40	70	6.5
DEAE-Sepharose	17	40	5.50	140	70	22.6
Reactive Red-agarose	6	6	2.28	380	30	61.3
F.p.l.c. (Mono Q)	1	2	1.34	670	17	108.1

found no inhibition at all with phenylmethanesulphonyl fluoride (1 mM). Taking advantage of the hydrophobic character of the enzyme, we used phenyl-Sepharose at first. Screening of different dye ligand affinity matrices led to the choice of Reactive Red-agarose. However, this step proved to be only useful in the absence of pyridoxal 5'-phosphate. Indeed, in the presence of pyridoxal 5'-phosphate the synthase was eluted very early and with a low specific activity. If pyridoxal 5'-phosphate was omitted from the eluting buffer, the 50 %-pure enzyme was recovered as an apoenzyme. This suggests that the red dye binds at or near the pyridoxal 5'-phosphate-binding site. The f.p.l.c. Mono Q step yielded an essentially homogeneous enzyme as judged by the electrophoresis data (Fig. 1). The pure enzyme was kept at 0 °C (freezing-thawing cycles should be avoided since the enzyme tends to be precipitated during such treatments) for a few months without any loss of activity.

Molecular-mass analysis

SDS/PAGE analysis of the purified enzyme showed a single band at 41 kDa in accordance with the molecular mass deduced

from the *bioF* gene sequence [16]. On gel-permeation chromatography (Sephadex G-100) the native enzyme was eluted as a single peak at 46 kDa, suggesting a monomeric structure for the active AOP synthase.

Amino acid composition and isoelectric point

N-Terminal sequencing (14 cycles) of the pure synthase gave the sequence, MNDRFRRELQVIEE, which was in accordance with the predicted sequence [16]. The amino acid composition was also consistent (within 10 %) with that predicted from the gene (Table 2). Isoelectrofocusing of the pure enzyme with a 5–7 pH gradient gave a band centred at pH 5.2 (results not shown).

Spectroscopic properties

The u.v.–visible spectrum of the AOP synthase is shown in Fig. 2 together with the spectrum of the apoenzyme collected after the dye ligand affinity step. This inactive apoenzyme (only if pyridoxal 5'-phosphate was omitted from the assay) did not show any characteristic absorbance. Activity and absorbance at 425 nm (attributed to the pyridoxal 5'-phosphate–enzyme imine) were recovered after dialysis in the presence of pyridoxal 5'-phosphate, confirming the absolute requirement for this cofactor.

Catalytic properties

As expected the purified holoenzyme was active even in the absence of pyridoxal 5'-phosphate. However, a 20 % enhancement of the specific activity was observed when 20 μ M-pyridoxal 5'-phosphate was included in the assay; it did not increase further at higher concentration. Nevertheless a saturating concentration (100 μ M) was used throughout the purification and in the assays to prevent any dissociation of the cofactor from the enzyme. Steady-state kinetic parameters were determined by using the direct linear plot [17]. With a saturating concentration of pimeloyl-CoA (150 μ M) an apparent K_m of 3 mM was found for L-alanine (concentration range 1–20 mM). For pimeloyl-CoA (concentration range 0.5–10 μ M), in the presence of 50 mM-L-alanine, a low apparent K_m of 1 μ M was found. In this case, because of the large consumption of pimeloyl-CoA during the assay (20–30 %), we used the approximation proposed by Lee & Wilson [18]. In these conditions the turnover number is 0.5 s⁻¹, giving a $k_{cat.}/K_m = 5 \times 10^5$ M⁻¹·s⁻¹ for pimeloyl-CoA.

Table 2. Amino acid composition of the purified AOP synthase

Amino acid	Composition (mol of residue/mol of protein)	
	Found	Predicted
D+N	38.12	35
E+Q	38.54	38
S	32.70	32
G	33.84	37
H	8.13	7
R	20.17	18
T	23.24	22
A	33.24	32
P	8.19	9
Y	10.00	9
V	19.39	21
M	9.82	11
C	Not determined	2
I	34.26	35
L	32.76	34
F	18.43	19
K	31.19	31

Fig. 2. U.v.–visible spectrum of the AOP synthase

(a) Pure AOP synthase in 10 mM-Tris/HCl buffer, pH 7.6; (b) spectrum of the apoenzyme recovered after the dye ligand affinity step (same buffer).

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