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Investigation of the first step of biotin biosynthesis in *Bacillus sphaericus*

Purification and characterization of the pimeloyl-CoA synthase, and uptake of pimelate

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The pimeloyl-CoA synthase from *Bacillus sphaericus* has been purified to homogeneity from an overproducing strain of *Escherichia coli*. The purification yielded milligram quantities of the synthase with a specific activity of 1 unit/mg of protein. Analysis of the products showed that this enzyme catalysed the transformation of pimelate into pimeloyl-CoA with concomitant hydrolysis of ATP to AMP. Using a continuous spectrophotometric assay, we have examined the catalytic properties of the pure enzyme. The pH profile under $V_{max.}$ conditions showed a maximum around 8.5. Apparent K_m values for pimelate, CoASH, ATP·Mg^{2-} and Mg^{2+} were respectively 145 μM, 33 μM, 170 μM and 2.3 mM. The enzyme was inhibited by Mg^{2+} above 10 mM. This acid-CoA ligase exhibited a very sharp substrate specificity, e.g. neither GTP nor pimelate analogues (di- or mono-carboxylic acids) were processed. The bivalent metal ion requirement was also investigated: Mn^{2+} (73 %) and Co^{2+} (32 %) but not Ca^{2+} could replace Mg^{2+}. The enzyme was inhibited by metal chelators such as 1,10-phenanthroline and EDTA. The synthase was a homodimer with a 28000-M_r subunit. *N*-Terminal sequencing definitely proved that this enzyme was encoded by the *bioW* gene. A careful study of pimelate uptake by *B. sphaericus*, *E. coli* and *Pseudomonas denitrificans* showed that this metabolite crossed the membrane of these micro-organisms by passive diffusion, ruling out the involvement of the *bioX* gene product as pimelate carrier.

INTRODUCTION

The metabolic pathway leading to biotin has been elucidated in *Escherichia coli* and *Bacillus sphaericus* [1,2]. The first common intermediate of this pathway, in these two bacteria, is pimeloyl-CoA (Scheme 1). Indeed, the transformation of pimeloyl-CoA to 8-amino-7-oxononanoate has been demonstrated in cell-free extracts of *E. coli* [3], *B. sphaericus* [4] and other bacteria [5], and we have recently reported the first purification to homogeneity of the 8-amino-7-oxononanoate synthase (EC 2.3.1.47) [6]. On the other hand, the origin of pimeloyl-CoA is not clear in all the bacteria so far studied. Izumi and coworkers have shown that a pimeloyl-CoA synthase activity, using pimelate as substrate, could be detected in various cell-free extracts [7], including those from *E. coli*, using a coupled assay with 8-amino-7-oxononanoate synthase. They also partially characterized this activity in *Bacillus megaterium* using a partially purified (34-fold) preparation [8,9]. However, the presence of this enzyme in *E. coli* has been questioned by several groups [1,10].

We have elucidated and sequenced the *bio* genes in *B. sphaericus* and showed that all the activities required for the transformation of pimelate into 8-amino-7-oxononanoate were encoded by the *bioXWF* cluster [10]. Although *bioF* was identified as the 8-amino-7-oxononanoate synthase gene, confirmed by the characterization of the pure synthase, initial genetic experiments suggested that *bioW* was involved in pimeloyl-CoA biosynthesis, i.e. a weak complementation was observed when *bioC* and *bioH* *E. coli* mutants, transformed with a plasmid harbouring the *bioW* gene, were grown on pimelate-supplemented medium [10]. No specific role could be attributed to *bioX*, although the hydrophobic character of the encoded protein suggested a membrane-bound protein (a permease for pimelate?).

To clarify these points, we have purified the pimeloyl-CoA synthase from *B. sphaericus*, and characterized it. We have also investigated pimelate uptake by *B. sphaericus* and other micro-organisms.

EXPERIMENTAL

Materials

Phenyl-Sepharose CL-4B, DEAE-Sepharose CL-6B and Reactive-Red 120–Agarose 3000-CL were purchased from Sigma (St. Louis, MO, U.S.A.). Sephadex G-100 was from Pharmacia (Uppsala, Sweden). All microbiological reagents were from Difco (Detroit, MI, U.S.A.). All chemicals were of the highest purity available and were purchased either from Sigma, Prolabo (Paris, France) or Aldrich (Milwaukee, WI, U.S.A.). $Na^{14}CN$ (45 mCi/mmol) was purchased from CEA (Saclay, France). [^{14}C]Inulin (3 mCi/g) was obtained from Sigma.

Strains

E. coli K 12 CIP 54.117 and *Pseudomonas denitrificans* CIP 63.54 were from the Institut Pasteur collection (Paris, France). *B. sphaericus* IFO 3525 [7] was a gift from Professor Y. Izumi. *E. coli* C268 (Sm^R, Δ*bioA*, *his*$^+$) [11] was a gift from Dr. A. Campbell. *E. coli* C268/pTG3403 was constructed at Transgene (R. Gloeckler, unpublished work).

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 FTX 90Q spectrometer (Tokyo, Japan). U.v./visible absorbance measurements were recorded on an Uvikon-930 Kontron (Rotkreuz, Switzerland) spectrometer.

Abbreviations used: Ches, 2-(cyclohexylamino)ethanesulphonic acid; PLP, pyridoxal 5′-phosphate; TEAP, triethylamine phosphate buffer.

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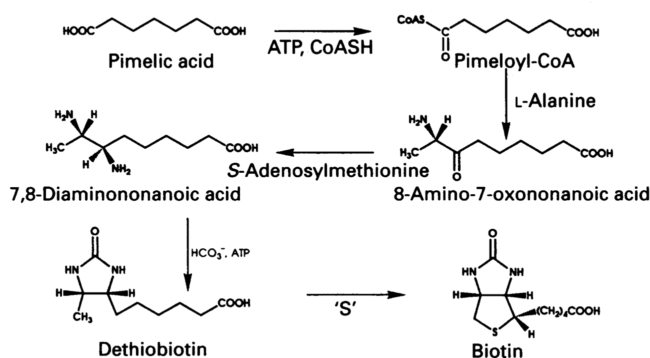


Scheme 1. Biotin biosynthesis in micro-organisms

Electrophoresis was performed on a Bio-Rad (Richmond, CA, U.S.A.) apparatus. Sonication was performed on a 150 W MSE (Crawley, Sussex, U.K.) ultrasonic disintegrator. Centrifugations were run on a Sorvall RC5 (Du Pont, Newton, CN, U.S.A.) centrifuge. Liquid scintillation countings were performed on a 1214 Pharmacia-LKB Wallac instrument. Radioactive t.l.c. plates (SiO_2) were scanned on an automatic t.l.c. linear analyser (Tracemaster 20) from Berthold (Berlin, Germany).

Syntheses

[1,7-^{14}C]Pimelic acid (1). Starting from 459 mg (2 mmol) of 1,5-dibromopentane and 2 mmol of $Na^{14}CN$ (5 mCi/mmol) we obtained compound 1 as described by Schimdt *et al.* [12]. Compound 1 was purified by anion-exchange chromatography (Dowex Bio-Rad 1X4, formate; 1.6 cm × 18 cm) using a linear formic acid gradient (0–0.5 M). Radiochemical yield was 65 %. The specific activity of compound 1 was assumed to be 5 mCi/mmol. ^{1}H n.m.r. (90 MHz): δ (p.p.m.) ($C^2H_3O^2H$) 1.2–1.9 (6 H, broad, CH_2), 2.4 (4 H, t, CH_2COO). On t.l.c. in chloroform/methanol/acetic acid (92: 8: 3, by vol.), one radioactive spot occurred at $R_F = 0.46$. Compound 1 was further characterized as its 4-methyl-7-methoxycoumarin diester (2) as described by Elbert *et al.* [13]. Treatment of compound 1 with excess of 4-bromomethyl-7-methoxycoumarin yielded one radioactive product which co-eluted with compound 2 using reverse-phase h.p.l.c. [LiChrosorb RP-18 7 μm-diam. column (Merck); 62.5 % (v/v) methanol in water, at a flow rate of 1.5 ml/min; retention time 11.5 min].

Di(4-methyl-7-methoxycoumarin)pimelate (2). This compound was obtained as already described [13]. M.p. 120–122 °C (literature value [13] 118–120 °C). ^{1}H n.m.r. (90 MHz): δ (p.p.m.) C^2HCl_3, tetramethylsilane) 1.2–1.9 (6 H, broad, CH_2), 2.5 (4 H, 5, CH_2CO), 3.85 (6 H, s, OCH_3), 5.25 (4 H, s, CH_2O), 6.3–7.5 (8 H, vinylic and aromatic protons).

Pimeloyl-CoA and 8-amino-7-oxononanoic acid were synthesized as already described [6].

Assays

Pimeloyl-CoA was colorimetrically determined in the form of its hydroxamate as already described [6] or at 230 nm (ϵ_{230} 4500 $M^{-1} \cdot cm^{-1}$) [14]. The CoASH concentration was determined at 260 nm (ϵ_{260} 16800 $M^{-1} \cdot cm^{-1}$), and the ATP concentration at 260 nm (ϵ_{260} 15400 $M^{-1} \cdot cm^{-1}$).

Pimeloyl-CoA synthase was assayed using two different procedures. Procedure A involved a coupled assay with 8-amino-7-oxononanoate synthase as described by Izumi *et al.* [9], but with modifications. The assay mixture consisted of 1.5 mM-disodium pimelate, 0.1 mM-CoASH, 2.0 mM-ATP, 10.0 mM-$MgCl_2$, 25.0 mM-L-alanine, 0.1 mM-pyridoxal phosphate (PLP), 0.1 m-unit of 8-amino-7-oxononanoate synthase (purified as previously described [6]), 0.2 M-NaCl, 50 mM-Tris/HCl, pH 8.5, in a total volume of 45 μl. The Tris buffer was carefully de-aerated to avoid CoASH oxidation. The reaction was initiated by the addition of pimeloyl-CoA synthase (5 μl), after which the mixture was rapidly vortexed, and incubated at 30 °C. The reaction was quenched at various times (30 s to 10 min) by the addition of trichloroacetic acid [5 % (w/v) final concentration]. 8-Amino-7-oxononanoate concentration was then determined using the *Saccharomyces cerevisiae* bioassay [6].

Procedure B: this determination is based on the appearance of the thioester bond at 230 nm (ϵ_{230} 4500 $M^{-1} \cdot cm^{-1}$) [14]. The quartz cuvette contained 1.5 mM-disodium pimelate, 0.1 mM-CoASH, 0.4 mM-ATP, 10.0 mM-$MgCl_2$, 0.2 M-NaCl, 50 mM-Tris/HCl, pH 8.5, (de-aerated) in a total volume of 400 μl. The reaction was initiated by the addition of pimeloyl-CoA synthase (5 μl) and the absorbance at 230 nm was recorded as a function of time, at 30 °C.

One unit is defined as the amount of enzyme which catalyses the formation of 1 μmol of pimeloyl-CoA per min in the conditions described for procedure B.

Protein assay

Protein concentrations were determined by the method of Bradford [15] using the reagent supplied by Bio-Rad. BSA fraction V, from Sigma, was used as a standard.

Identification of the products

The reaction mixture was as described above for procedure B. After the absorbance at 230 nm had reached a constant value (ΔA_{230} 0.45), the products (25 μl of the mixture) were separated by reverse-phase h.p.l.c. [LiChrospher 100 RP-8 5 μm (Merck), detection at 260 nm, flow rate of 1 ml/min]. For the separation of ATP, ADP and AMP, we used a linear gradient (15 min) from 1 % acetonitrile in 0.2 M-triethylamine phosphate buffer (TEAP), pH 5.5, to 5 % acetonitrile in 0.2 M-TEAP, pH 5. For the identification of pimeloyl-CoA we used a linear gradient (20 min) from 6 % acetonitrile in 0.2 M-TEAP, pH 5.5, to 12 % acetonitrile in 0.2 M-TEAP, pH 5.5. These identifications were confirmed by the use of an anion-exchange column (f.p.l.c. MonoQ HR 5/5, Pharmacia): detection at 260 nm, flow rate of 1 ml/min, linear KCl gradient (0–0.5 M, 15 min) in 40 mM-potassium phosphate buffer, pH 7.0.

Culture

E. coli C268/pTG3403 was grown in Erlenmeyer flasks (250 ml batches) at 37 °C in the following medium (per litre): K_2HPO_4 1 g, KCl 0.5 g, $MgSO_4$,$7H_2O$ 0.5 g, $FeSO_4$,$7H_2O$ 10 mg, $MnSO_4$,$7H_2O$ 10 mg, Proteose Peptone 10 g, Casamino Acids 3 g, thiamin HCl 0.02 mg, glycerol 20 g, pH 6.8. Ampicillin was sterilized by filtration and added at a final concentration of 0.1 mg/ml. Cells were harvested at early stationary phase by centrifugation (4000 *g*, 15 min) and washed with 0.4 M-NaCl, 50 mM-KH_2PO_4, pH 7.0, 50 mM-$MgSO_4$,$7H_2O$. After centrifugation (4000 *g*, 15 min) cells were 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-Tris/HCl, pH 8.0, 5 mM-2-mercaptoethanol, 20 % (v/v) glycerol, 1 mM-EDTA, 1 mM-phenylmethanesulphonyl fluoride, 1 μM-pepstatin A) and disrupted by sonication for 5 min (five 1 min pulses with intermittent 1 min cooling periods). The cellular debris were removed by centrifugation (27000 *g*, 20 min).

Precipitation. The supernatant was made up to an ammonium sulphate concentration of 1.4 M by the slow addition of crystals, and then stirred for 30 min. After centrifugation (27000 *g*, 15 min) the supernatant was made up to 2.8 M-ammonium sulphate by the further addition of crystals, stirred for 30 min and centrifuged (27000 *g*, 15 min). The pellet was dissolved in 50 ml of buffer B [20 mM-Tris/HCl, pH 8.0, 5 mM-2-mercaptoethanol, 20% (v/v) glycerol, 1 mM-EDTA, 3 M-NaCl].

Phenyl-Sepharose. The enzyme solution was loaded onto a phenyl-Sepharose column (2.5 cm × 18 cm) equilibrated with buffer B. The proteins were eluted using a linear gradient (3–0 M-NaCl in buffer B; 2 × 200 ml) followed by a wash with 100 ml of buffer C (buffer B without NaCl). Active fractions were pooled.

Reactive-Red–Agarose. The enzyme solution was loaded on to a Reactive-Red–Agarose column (2.5 cm × 10 cm) equilibrated with buffer C. After washing the column with 100 ml of buffer C the proteins were eluted using a linear salt gradient (0–4 M-NaCl in buffer C; 2 × 200 ml). Active fractions were pooled, concentrated by ultrafiltration (Amicon P 10) and dialysed against buffer D (20 mM-Tris/HCl, pH 8.0, 5 mM-2-mercaptoethanol, 1 mM-EDTA).

F.p.l.c. on Mono Q. The enzyme was purified by portions on a Mono Q HR 5/5 f.p.l.c. (Pharmacia) column using a biphasic linear salt gradient (0–0.15–0.4 mM-NaCl in 10 mM-Tris/HCl, pH 8.0). Active fractions were pooled and concentrated (centriprep 10 Amicon).

F.p.l.c. on Superose 12. The enzyme was finally purified by gel filtration on an f.p.l.c. Superose 12 HR 10/30 (Pharmacia) column using buffer C as the solvent. The enzyme was stored at −20 °C without loss of activity.

M_r determination

The subunit M_r was determined by SDS/PAGE [16] (15%) with the following M_r standards: BSA 66000, ovalbumin 45000, glyceraldehyde-3-phosphate dehydrogenase 36000, carbonic anhydrase 29000, trypsinogen 24000, trypsin inhibitor 20100, α-lactalbumin 14200. The proteins were stained with Coomassie Brillant Blue R-250. The native M_r was determined by gel permeation chromatography on a Sephadex G-100 column (1.6 cm × 80 cm) using 20 mM-potassium phosphate, pH 7.0, as the solvent. The M_r standards utilized were the following: Blue Dextran, void volume; alcohol dehydrogenase, 150000; BSA, 66000; carbonic anhydrase, 29000; cytochrome *c*, 12400; aprotinin, 6500.

N-Terminal sequence determination

Sequencing was performed by Dr. J. P. Le Caer (CNRS, Gif sur Yvette, France) on an Applied Biosystems apparatus (Foster City, CA, U.S.A.).

Isoelectrofocusing

The isoelectric point was determined on a polyacrylamide gel using a 3–10 pH gradient (Biolyte from Bio-Rad). The bands were stained with Crocein Scarlet 7B and Coomassie Brillant Blue R-250. Standards were from Bio-Rad.

Uptake experiments

B. sphaericus and *E. coli* were grown to mid-exponential phase at 37 °C, in LB [17], GP [7] or M9 [17] medium supplemented with 2 g of Casamino Acids/l. Disodium pimelate was at a concentration of 0.5 g/l when added. *P. denitrificans* was grown to mid-exponential phase at 30 °C, in (per litre): Bactopeptone 20 g, $MgCl_2,6H_2O$ 1.4 g, K_2SO_4 10 g, glycerol 10 g, pH 7.0. The cells were collected by centrifugation (4000 *g* for 10 min) and washed twice with solution A (0.4 M-NaCl, 50 mM-KH_2PO_4, pH 7.0, 50 mM-$MgSO_4$). In all cases the cells were resuspended in sufficient M9 medium without glucose and without Casamino-Acids, but supplemented with 100 μg of chloramphenicol/ml, so that the cell content was 4×10^9 cells/ml. Glucose was at 2% (w/v) and glycerol at 3% (v/v) when added.

The uptake was started by the addition of [^{14}C]pimelate and the cells were gently shaken at 37 °C (30 °C for *P. denitrificans*). Control experiments were performed with boiled cells (10 min at 100 °C). Aliquots (100 μl) were removed at various times and rapidly filtered on a cellulose acetate filter (0.45 μm pore size; Sartorius). The filter was then immediately washed three times with 3 ml of warm solution A, dried at 60 °C and counted for radioactivity in 10 ml of scintillation liquid (Aquasol-2 from Du Pont). Countings were corrected for quenching by the external standard method.

The intracellular volume of *B. sphaericus* was measured using [^{14}C]inulin as described for *E. coli* by Winkler & Wilson [18].

RESULTS AND DISCUSSION

Assays

Izumi *et al.* [9] have described a discontinuous coupled assay for pimeloyl-CoA synthase using 8-amino-7-oxononanoate synthase. Although very sensitive (in the picomolar range), the microbiological determination of 8-amino-7-oxononanoate is tedious and not very accurate [6]. Indeed, the growth of the test organism requires at least 15 h and the manual measurement of growth diameters limits its accuracy [6]. We have thus used a continuous assay for the pimeloyl-CoA synthase based on the appearance of the thioester-bond chromophore (ϵ_{230} 4500 $M^{-1} \cdot cm^{-1}$) which is less sensitive than the former but more accurate and much easier to set up. Using this assay, progress curves were linear over 5 min and the calculated initial velocities showed a linear response over a wide range of enzyme concentrations (results not shown). This assay was used throughout this study unless otherwise stated.

Purification

The *B. sphaericus* pimeloyl-CoA synthase was purified from an overproducing strain, *E. coli* C268, containing the synthase-cloned gene downstream of a lactose promoter inserted into plasmid pTG3403 (R. Gloeckler, unpublished work). Overproduction led to about 2% of the soluble proteins in *E. coli* being the synthase. Table 1 summarizes the purification, which permitted the isolation of the enzyme in milligram quantities with a specific activity of about 1 unit/mg. This value is at least 1000-fold higher than that reported for the *B. megaterium* enzyme preparation, which was not homogeneous [9].

Glycerol, EDTA and 2-mercaptoethanol efficiently stabilized the enzyme, which showed a slow inactivation in their absence, and thus were used throughout the purification. The Phenyl-Sepharose/Reactive-Red sequence was very efficient (see Table 1) leaving only small contaminants which were removed using the f.p.l.c. columns, yielding an electrophoretically pure enzyme (Fig. 1). However, we observed a poor recovery after the f.p.l.c. columns, probably owing to non-specific hydrophobic interactions on the matrices.

Product analysis

Analysis of the products using reverse-phase h.p.l.c. and anion-exchange chromatography (f.p.l.c.) showed the presence of pimeloyl-CoA and AMP. No ADP could be detected. The reaction can thus be written as shown in Scheme 2 and the enzyme classified as an acid-CoA ligase (AMP-forming). The 1984 edition of the IUB Enzyme Nomenclature [24] has classified this enzyme as the 6-carbohexanoate-CoA ligase (EC 6.2.1.14)

Table 1. Summary of the purification of the pimeloyl-CoA synthase

Step	Volume (ml)	Protein (mg)	Activity (units)	Specific activity (units/mg)	Yield (%)	Purification (fold)
Crude extract*	110	1350	†	–	–	–
Ammonium sulphate precipitation	56	1043	24.6	0.024	–	–
Phenyl-Sepharose	50	176	26.5	0.15	(100)	6
Reactive-Red–Agarose	38	38.5	20.4	0.53	77	22
F.p.l.c. Mono Q	11	10.3	8.9	0.93	34	39
F.p.l.c. Superose	5	4.8	4.8	1.00	18	42

* Starting from 2.5 l of *E. coli* culture.
† Activity could not be determined because of high absorption at 230 nm.

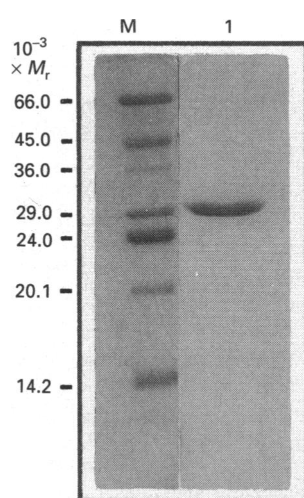


Fig. 1. SDS/PAGE of the pure pimeloyl-CoA synthase

M, M_r standards (see the Experimental section); 1, 20 μg of the pure synthase was loaded.

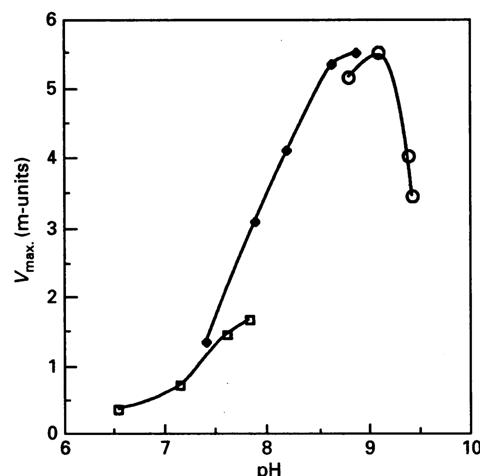
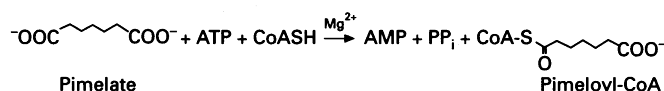


Fig. 2. pH profile of the pimeloyl-CoA synthase activity

Conditions were as described under the Experimental section, except that the buffers used were the following: □, 50 mM-potassium phosphate; ◆, 50 mM-Tris/HCl; ○, 50 mM-glycine/NaOH. Ionic strength was kept constant (0.25 M) by the addition of KCl.



Scheme 2. Reaction catalysed by pimeloyl-CoA synthase

[systematic nomenclature: 6-carbohexanoate:CoA ligase (AMP-forming)], on the basis of the work of Izumi *et al.* [7,8]. However, a careful reading of these reports reveals that there is no evidence at all for the formation of ADP or AMP upon hydrolysis. To our knowledge the results reported here are the first evidence for a systematic classification. Fortunately, the nomenclature proposed in 1984 is correct.

Catalytic properties

The pH profile of the catalysis under $V_{max.}$ conditions was bell-shaped with a maximum around pH 8.5–9.0 (Fig. 2). No activation was detected using different buffers [Tris/HCl, 2-(cyclohexylamino)ethanesulphonic acid (Ches)/HCl or glycine/NaOH] although borate buffer slightly inactivated the enzyme.

Steady-state kinetic parameters were determined using the direct linear plot [19] (Fig. 3). Experimental conditions were as described for procedure B, i.e. 1.5 mM-pimelate, 0.1 mM-CoASH, 0.4 mM-ATP and 10.0 mM-Mg^{2+} except when varied. Apparent K_m values for pimelate, ATP·Mg^{2-}, CoASH were respectively 145 μM, 170 μM and 33 μM. For the K_m determination of CoASH, because the progress curves were not linear when the CoASH concentration was less than 50 μM, we used the approximation proposed by Lee & Wilson [20]. To keep the Mg^{2+} concentration constant when the ATP·Mg^{2-} concentration was varied, a constant excess of 10 mM-$MgCl_2$ over the ATP concentration was used [21]. When the Mg^{2+} concentration was varied from 2 mM to 10 mM, conditions in which the ATP·Mg^{2-} concentration is constant [21], an apparent K_m of 2.3 mM was found, but, as already noted for the *B. megaterium* enzyme [9], inhibition was observed at higher Mg^{2+} concentrations. Assuming an apparent $V_{max.}$ of 1 unit/mg we calculated a $k_{cat.}$ of 0.4 s^{-1}, giving an apparent $k_{cat.}/K_m$ of 3×10^3 $M^{-1} \cdot s^{-1}$, for pimelate.

Specificity

The specificity of the synthase was examined using nucleotide and pimelate analogues and various bivalent cations (Table 2). GTP was a very poor substrate for the pimeloyl-CoA synthase and, as expected, no activity was observed using AMP. Izumi *et al.* reported that ADP could replace ATP in the reaction catalysed by the *B. megaterium* enzyme (relative activity 80 %) [9], but we found it inactive towards the *B. sphaericus* enzyme. Contrary to findings with other acid-CoA ligases [22], the pimeloyl-CoA

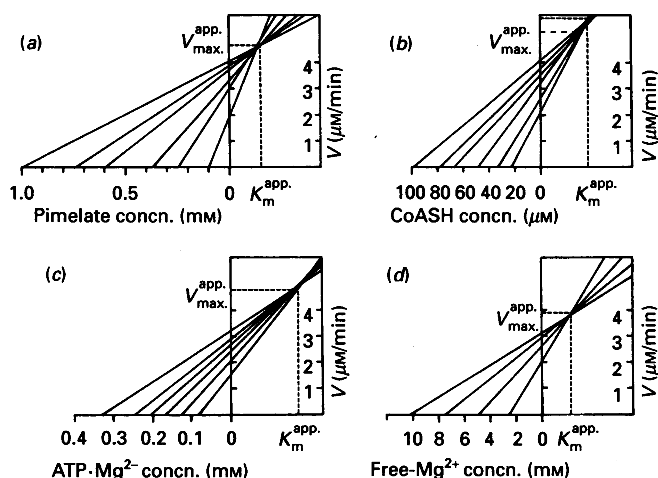


Fig. 3. Estimation of the kinetic parameters by the direct linear plot

Initial velocities were determined using procedure B. (*a*) Pimelate concentration was varied; 1.6 m-units of enzyme was used in each assay; $K_m^{app.} = 145\,\mu M$; $V_{max.}^{app.} = 4.7\,\mu M/min$. (*b*) CoASH concentration was varied; 1.6 m-units of enzyme was used in each assay; $K_m^{app.} = 33\,\mu M$; $V_{max.}^{app.} = 5.7\,\mu M/min$. (*c*) $ATP \cdot Mg^{2-}$ concentration was varied; 1.3 m-units of enzyme was used in each assay; $K_m^{app.} = 170\,\mu M$; $V_{max.}^{app.} = 4.8\,\mu M/min$. (*d*) Free Mg^{2+} concentration was varied; 1.3 m-units of enzyme was used in each assay; $K_m^{app.} = 2.3\,mM$; $V_{max.}^{app.} = 3.9\,\mu M/min$.

Table 2. Substrate specificity of the pimeloyl-CoA synthase

Conditions were as described in the Experimental section for procedure B, except that the analogue was used instead of the substrate.

Substrate/analogue	Concentration used (mM)	Relative activity (%)
ATP	0.4	100
GTP	0.4	0.4
ADP	0.4	< 0.1
AMP	0.4	< 0.1
Pimelate	1.5	100
Succinate	10	< 0.1
Glutarate	10	< 0.1
Adipate	10	2
Suberate	10	< 0.1
Heptanoate	10	< 0.1
Hexanoate	10	< 0.1
Mg^{2+}	10	100
Mn^{2+}*	10	73
Co^{2+}*	10	32
Ca^{2+}	10	< 0.5

* Procedure A was used and results were compared with those obtained using Mg^{2+} in the same conditions.

synthase did not accept alternative substrates for pimelate. Among the dicarboxylic acids (C_4–C_8) we only detected some activity with adipic acid. None of the monocarboxylic acids we tested (C_6 and C_7) was a suitable substrate. This sharp specificity suggests a unique role for the pimeloyl-CoA synthase, i.e. biotin biosynthesis. The requirement for bivalent metal ions was also examined. Using the coupled assay with the 8-amino-7-oxononanoate synthase (because of high absorption at 230 nm) we found that Mn^{2+}, and to a lesser extent Co^{2+}, could replace Mg^{2+}. No activity was observed with Ca^{2+}. As noted for the *B.*

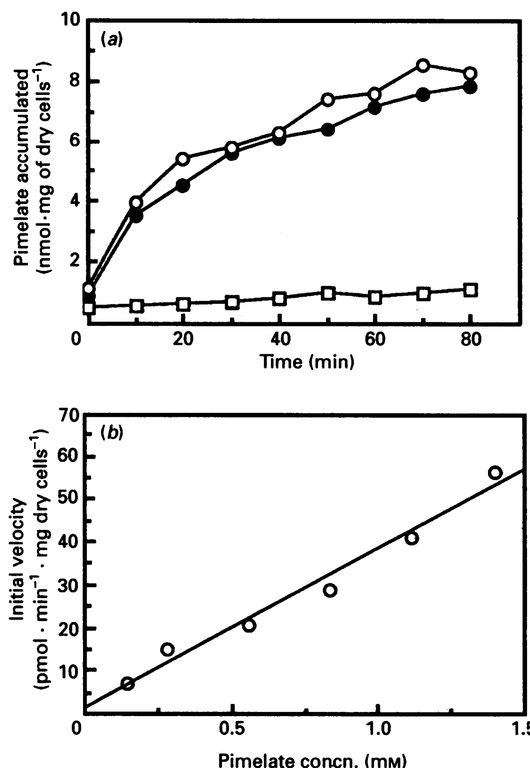


Fig. 4. Pimelate uptake by *Bacillus sphaericus*

(*a*) Time course of pimelate (3.2 mM) uptake by *B. sphaericus*. □, Control experiment with boiled cells; ●, uptake in the absence of energy source; ○, uptake in the presence of glucose. The addition of glycerol gave similar results (see text). (*b*) Effect of pimelate concentration on the initial velocities measured during the first 5 min of the uptake. The linearity was observed at concentrations up to 20 mM (results not shown).

megaterium enzyme [9], metal chelators such as EDTA and 1,10-phenanthroline were inhibitors of the pimeloyl-CoA synthase: 50% inhibition at 10 mM for EDTA and 50% inhibition at 25 μM for 1,10-phenanthroline.

Molecular properties

SDS/PAGE analysis of the purified enzyme (Fig. 1) showed a single band with an M_r of 28000, in accordance with the molecular mass deduced from the *bioW* gene sequence [10]. On gel-permeation chromatography (Sephadex G-100) the native enzyme was eluted as a single peak at M_r 60000, suggesting a homodimeric structure for the active synthase. The u.v./visible spectrum of the pure enzyme did not reveal the presence of any chromophore or cofactor. Isoelectrofocusing of the pure enzyme using a 5–7 pH gradient gave a band centred at pH 6.8 (results not shown).

N-Terminal sequence

N-Terminal sequencing (15 cycles: MLICYSIRMRAAEKN) confirmed the purity of our preparation and is consistent with the sequence predicted from the *bioW* gene [10]. This result, together with the molecular mass analysis, definitely proves that the pimeloyl-CoA synthase is encoded by the *bioW* gene. Thus the *bioX* gene, immediately upstream of *bioW*, is not involved in the transformation of pimelate into pimeloyl-CoA as we originally assumed. The sequence of the pimeloyl-CoA synthase was compared with the NBRF-PIR library but no significantly similar proteins were found. Sequence analysis of the protein encoded by *bioX* showed a strong hydrophobic character consistent with a

membrane-bound protein. This prompted us to investigate pimelate uptake by *B. sphaericus*, as the *bioX* gene product could be a permease.

Pimelate uptake

This problem was first addressed by Pai & McLaughlin in 1960 using *E. coli* K12 and *P. denitrificans* cells [23]. These authors noted that whereas *E. coli* cells were not permeable to pimelate, *P. denitrificans* showed a significant uptake. Whether this uptake was processed by a permease and is therefore energy-dependent was not investigated. We thus decided to check more precisely the permeability of *B. sphaericus*, *E. coli* and *P. denitrificans* cells to pimelate.

The time course of the uptake by *B. sphaericus* is shown in Fig. 4. Although this process was slow, a significant plateau was reached after 1 h. Assuming a volume of 2.6 μl per mg of dry weight for *B. sphaericus*, the internal concentration of pimelate reached after 1 h was close to the external concentration. Neither the rate nor the steady-state plateau was modified by the addition of energy sources (glucose or glycerol). Furthermore, induction of a putative permease was not detected when the cells were grown in the presence of pimelate as an alternative carbon source or in different growth media (LB, GP). Initial velocities measured during the first minutes of the uptake showed a linear dependence on the pimelate concentration over a wide range (up to 20 mM). This suggests the absence of any permease for pimelate and that this metabolite probably crosses the membrane by passive diffusion.

Pimelate uptake was also investigated in *E. coli* cells. We confirmed that the uptake was not significant with *E. coli* K12 or *E. coli* C268: after 1 h the radioactivity accumulated by the cells was not significantly higher than the background. When *E. coli* C268, transformed with a plasmid bearing the *bioXWF* cluster under the tetracycline promoter (pTG1446; R. Gloeckler, unpublished work), was used, the uptake was not enhanced. However, when grown in the presence of exogenous pimelate *E. coli* C268/pTG1446 was able to metabolize this compound into 8-amino-7-oxononanoate, confirming a slow but real uptake (O. Ploux & R. Gloeckler, unpublished work).

Finally, pimelate uptake was studied in *P. denitrificans*. We confirmed that the uptake was faster as compared with *E. coli* and *B. sphaericus* (the steady-state plateau was reached within 10 min). Initial velocity studies showed that the process was passive diffusion (no energy dependence and no saturation kinetics; results not shown).

In conclusion, pimelate crosses the membrane of these different bacteria by passive diffusion without involving any carrier protein. This rules out our second hypothesis concerning the role of the *bioX* gene product. Whether this gene is involved in pimelate biosynthesis remains to be established by genetic studies and experiments *in vitro*.

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