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 Vmax 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 synthesis 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 microorganisms 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 microorganisms.

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.). Na14CN (45 mCi/mmol) was purchased from CEA (Saclay, France). [14C]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, ΔbioA, 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)ethanesulfonic acid; PLP, pyridoxal 5'-phosphate; TEAP, triethylenamine phosphate buffer.
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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 counting was performed on a 1214 \textsuperscript{14}C-pharmacacia-LKB Wallac instrument. Radioactive t.l.c. plates (SiO\textsubscript{2}) were scanned on an automatic t.l.c. linear analyser (Tracemaster 20) from Berthold (Berlin, Germany).

**Syntheses**

\textbf{[1,7-\textsuperscript{14}C]Pimelic acid (1)}: Starting from 459 mg (2 mmol) of 1,5-dibromopentane and 2 mmol of Na\textsuperscript{44}CN (5 mmCi/mmol) we obtained compound 1 as described by Schmid et al. [12]. Compound 1 was purified by anion-exchange chromatography (Dowex Bio-Rad 1X4, formate; 1.6 cm $\times$ 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. \textsuperscript{1}H n.m.r. (90 MHz): $\delta$ (p.p.m.) (CH$_2$OH)H 1.2-1.9 (6 H, broad, CH$_3$), 2.4 (4 H, t, CH$_2$COO). On t.l.c. in chloroform/methanol/acetic acid (92: 8: 3, by vol), one radioactive spot occurred at $R_p$ = 0.46. Compound 1 was further characterized as its 4-methyl-7-methoxyanmoni diester (2) as described by Elbert et al. [13]. Treatment of compound 1 with excess of 4-bromomethyl-7-methoxyanmoni yielding one radioactive product which co-eluted with compound 2 using reverse-phase h.p.l.c. [LiChrosorb RP-18 7\,\mu m-diam. column (Merck); 62.5\%, ($\vee$/$\nu$) methanol in water, at a flow rate of 1.5 ml/min; retention time 11.5 min].

\textbf{Di(4-methyl-7-methoxyanmoni) pimelate (2)}: This compound was obtained as already described [13]. M.p. 120-122 °C (literature value [13] 118-120 °C). \textsuperscript{1}H n.m.r. (90 MHz): $\delta$ (p.p.m.) CH$_3$Cl$_4$, tetramethylsilane) 1.2-1.9 (6 H, broad, CH$_3$), 2.5 (4 H, 5, CH$_3$CO), 3.85 (6 H, s, OCH$_3$), 5.25 (4 H, s, CH$_2$O), 6.3-7.5 (8 H, vinylic, and aromatic protons).

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

**Assays**

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

Pimelyol-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-alamine, 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 pimelyol-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 ($\epsilon_{230}$ 4500 m$^{-1}$ 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 pimelyol-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 pimelyol-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 ($A_{230}$ 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 pimelyol-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 (p.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**

\textit{E. coli} C268/pTG3403 was grown in Erlenmeyer flasks (250 ml batches) at 37 °C in the following medium (per litre): K$_2$HPO$_4$, 1 g; KCl, 0.5 g; MgSO$_4$$\cdot$7H$_2$O, 0.5 g; FeSO$_4$$\cdot$7H$_2$O, 10 mg; MnSO$_4$$\cdot$7H$_2$O, 10 mg; Proteose Peptone 10 g; Casamino Acids 3 g; thiamin HCl 0.02 mg; glycerol 20 g, pH 6.8. Ampicillin was sterilized by filtration and added at a final concentration of 0.1 mg/ml. Cells were harvested at early stationary phase by centrifugation (4000 g, 15 min) and washed with 0.4 m-NaCl, 50 mm-KH$_2$PO$_4$, pH 7.0, 50 mm-MgSO$_4$$\cdot$7H$_2$O. 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-phenylmethylsulphonyl fluoride, 1 µm-pestatin 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 mM-NaCl].

Phenyl-Sepharose. The enzyme solution was loaded onto a phenyl-Sepharose column (2.5 cm x 18 cm) equilibrated with buffer B. The proteins were eluted using a linear gradient (3–0 M-NaCl in buffer B; 2 x 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 x 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 x 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, determination

The subunit M, was determined by SDS/PAGE [16] (15%) with the following M, 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 Brilliant Blue R-250. The native M, was determined by gel permeation chromatography on a Sephadex G-100 column (1.6 cm x 80 cm) using 20 mM-potassium phosphate, pH 7.0, as the solvent. The M, 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.).

Isoelectric focusing

The isoelectric point was determined on a polycrylamide gel using a 3–10 pH gradient (Biolyte from Bio-Rad). The bands were stained with Cooitin Scarlet B and Coomassie Brilliant 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 Caseamino Acids/l. Disodium pimelate was at a concentration of 0.5 g/l when E. coli was grown to mid-exponential phase at 30 °C, in (per litre): Bactopeptone 20 g, MgCl₂·6H₂O 1.4 g, K₂SO₄ 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-KH₂PO₄, pH 7.0, 50 mM-MgSO₄). 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 x 10⁶ cells/ml. Glucose was at 2% (w/v) and glycerol at 3% (v/v) when added.

The uptake was started by the addition of [¹⁴C]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 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 [¹⁴C]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 (ε₅₂₀ 4500 M⁻¹ 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. Gloccker, 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-carboxyhexanoate-CoA ligase (EC 6.2.1.14)
Table 1. Summary of the purification of the pimeloyl-CoA synthase

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

* Starting from 2.5 ml 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, M₀ standards (see the Experimental section); 1, 20 μg of the pure synthase was loaded.

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\[
\begin{align*}
\text{Pimelate} & \xrightarrow{\text{ATP + CoASH + Mg}^{2+}} \text{AMP + PP}_i + \text{CoAS} \xrightarrow{\text{Mg}^{2+}} \text{Pimeloyl-CoA} \\
\text{OOC} & - \text{COO}^- + \text{ATP} + \text{CoASH} \xrightarrow{\text{Mg}^{2+}} \text{AMP + PP}_i + \text{CoAS} \xrightarrow{\text{Mg}^{2+}} \text{Pimeloyl-CoA}
\end{align*}
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Scheme 2. Reaction catalysed by pimeloyl-CoA synthase

[systematic nomenclature: 6-carboxhexanoate: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 \( V_{\text{max}} \) 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)ethanesulfonic 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 \( V_{\text{max}} \) of 1 unit/mg we calculated a \( k_{\text{cat}} \) of 0.4 s\(^{-1}\), giving an apparent \( k_{\text{cat}}/K_m \) of \( 3 \times 10^3 \) M\(^{-1}\)·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
Table 2. Substrate specificity of the pimeloyl-CoA synthase

<table>
<thead>
<tr>
<th>Substrate/analogue</th>
<th>Concentration used (mm)</th>
<th>Relative activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP</td>
<td>0.4</td>
<td>100</td>
</tr>
<tr>
<td>GTP</td>
<td>0.4</td>
<td>0.4</td>
</tr>
<tr>
<td>ADP</td>
<td>0.4</td>
<td>&lt; 0.1</td>
</tr>
<tr>
<td>AMP</td>
<td>0.4</td>
<td>&lt; 0.1</td>
</tr>
<tr>
<td>Pimelate</td>
<td>1.5</td>
<td>100</td>
</tr>
<tr>
<td>Succinate</td>
<td>10</td>
<td>&lt; 0.1</td>
</tr>
<tr>
<td>Glutarate</td>
<td>10</td>
<td>&lt; 0.1</td>
</tr>
<tr>
<td>Adipate</td>
<td>10</td>
<td>2</td>
</tr>
<tr>
<td>Suberate</td>
<td>10</td>
<td>&lt; 0.1</td>
</tr>
<tr>
<td>Heptanoate</td>
<td>10</td>
<td>&lt; 0.1</td>
</tr>
<tr>
<td>Hexanoate</td>
<td>10</td>
<td>&lt; 0.1</td>
</tr>
<tr>
<td>Mg²⁺</td>
<td>10</td>
<td>100</td>
</tr>
<tr>
<td>Mn²⁺</td>
<td>10</td>
<td>73</td>
</tr>
<tr>
<td>Co²⁺</td>
<td>10</td>
<td>32</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>10</td>
<td>&lt; 0.5</td>
</tr>
</tbody>
</table>

* Procedure A was used and results were compared with those obtained using Mg²⁺ in the same conditions.

The pimeloyl-CoA synthase did not accept alternative substrates for pimelate. Among the dicarboxylic acids (C₄-C₈) we only detected some activity with adipic acid. None of the monocarboxylic acids we tested (C₄ and C₆) 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 Mn²⁺, and to a lesser extent Co²⁺, could replace Mg²⁺. No activity was observed with Ca²⁺. As noted for the BioX 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 Mₛ of 28,000, 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 Mₛ 60,000, 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. Isoelectric focusing 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 of 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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REFERENCES


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