Biochemical and Molecular Characterization of Phenylacetate-Coenzyme A Ligase, an Enzyme Catalyzing the First Step in Aerobic Metabolism of Phenylacetic Acid in *Azoarcus evansii*

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Phenylacetate-coenzyme A ligase (PA-CoA ligase; AMP forming, EC 6.2.1.30), the enzyme catalyzing the first step in the aerobic degradation of phenylacetate (PA) in *Azoarcus evansii*, has been purified and characterized. The gene (*paaK*) coding for this enzyme was cloned and sequenced. The enzyme catalyzes the reaction of PA with CoA and MgATP to yield phenylacetyl-CoA (PACoA) plus AMP plus PPI. The enzyme was specifically induced after aerobic growth in a chemically defined medium containing PA or phenylalanine (Phe) as the sole carbon source. Growth with 4-hydroxyphenylacetate, benzoate, adipate, or acetate did not induce the synthesis of this enzyme. This enzymatic activity was detected very early in the exponential phase of growth, and a maximal specific activity of 76 nmol min⁻¹ mg⁻¹ of cell protein⁻¹ was measured. After 117-fold purification to homogeneity, a specific activity of 48 µmol min⁻¹ mg⁻¹ of protein⁻¹ was achieved with a turnover number (catalytic constant) of 40 s⁻¹. The protein is a monomer of 52 kDa and shows high specificity towards PA; other aromatic or aliphatic acids were not used as substrates. The apparent *Kₐ* values for PA, ATP, and CoA were 14, 60, and 45 µM, respectively. The PA-CoA ligase has an optimum pH of 8 to 8.5 and a pI of 6.3. The enzyme is labile and requires the presence of glycerol for stabilization. The N-terminal amino acid sequence of the purified protein showed no homology with other reported PA-CoA ligases. The gene encoding this enzyme is 1,320 bp long and codes for a protein of 48.75 kDa (440 amino acids) which shows high similarity with other reported PA-CoA ligases. An amino acid consensus for an AMP binding motif (VX²SSGTTGXP) was identified.

The biochemical and molecular characteristics of this enzyme are quite different from those of the isoenzyme catalyzing the same reaction under anaerobic conditions in the same bacterium.

Phenylacetic acid and its mono- or dihydroxylated derivatives are formed in nature from a wide variety of natural as well as synthetic compounds. Their microbial biodegradation and biotransformation have received the attention of many research groups for a long time. A number of bacteria and fungi have been isolated and studied to reveal the metabolic pathways of these acids under both aerobic and anaerobic growth conditions.

Recently, the complete anaerobic metabolism of phenylacetic acid in the bacterium *Thauera aromatica* has been shown to follow a novel α-oxidation of the side chain of the coenzyme A (CoA)-activated acid, leading to the formation of the central metabolite benzoyl-CoA (Fig. 1). Further metabolism of benzoyl-CoA subsequently leads to the formation of three acetyl-CoA molecules and one CO₂ molecule (21, 22, 24, 31, 35, 38, 39).

It has been established that the aerobic metabolism of most aromatic compounds starts by ring hydroxylation reactions carried out by mono- and dioxygenases. These oxic reactions, which are widely distributed in microorganisms, bring the aromatic rings of many aromatic compounds to the redox state for further transformation has received the attention of many research groups (7, 8, 10, 15, 25, 41, 43). There-
energy source under both aerobic and anaerobic growth conditions. A PA-CoA ligase involved in the anaerobic metabolism of this acid has been purified and characterized from this bacterium (30). During aerobic growth of *A. evansii* with PA, a PA-CoA ligase activity which was consistent with the rate of substrate utilization by whole cells was detected. There was some doubt as to whether this activity was due to the enzyme catalyzing the same reaction under anaerobic conditions in the same bacterium or to another isoenzyme which is specifically induced under aerobic conditions.

In this report, I describe the purification, characterization, and gene sequence of the isoenzyme catalyzing the formation of PACoA under aerobic conditions in *A. evansii*.

**MATERIALS AND METHODS**

**Growth of bacteria.** *A. evansii* KB740 (DSM 6898 [3]) was grown under aerobic conditions in chemically defined medium on 5 mM PA, 4-hydroxyphenylacetate (4-OHPA), B, Phe, acetate, or adipate. The growth medium contained 40 mM potassium phosphate buffer (pH 7.4), 10 mM ammonium chloride, 0.1 mM calcium chloride, and 0.8 mM magnesium sulfate and was supplemented with SL-10 trace element solution and VL-7 vitamin solution as previously described (30). The medium was dispensed in Erlenmeyer flasks (300 ml/1-liter flask), inoculated, and incubated at 37°C with shaking (180 rpm). Anaerobic growth with PA plus nitrate, growth measurement, and cell harvest were as described previously (30). Growth on a large scale with phenylacetic acid was carried out in a 200-liter fermentor with stirring at 200 rpm and a flow of sterile air at 90 liters min\(^{-1}\). Cells were frozen in liquid nitrogen until used.

**Preparation of cell extracts.** Frozen or fresh cells were suspended in Tris-HCl buffer, pH 7.8 (1 g of cells/2 ml of buffer), in the presence of DNase I, 2 mM MgCl\(_2\), 2 mM dithioerythritol (DTE), and 20% (wt/vol) glycerol. The cell slurry was passed twice through a French pressure cell at 137 MPa, and the lysate was centrifuged at 4°C for 2 h at 100,000 \(\times\) g.

**CoA ligase activity.** The activation of the aromatic acids to their corresponding CoA thioesters by CoA ligases was monitored in a coupled enzyme assay as described before (30). This assay couples the formation of AMP or ADP, which results from CoA ester formation, to an ATP-regenerating system containing myokinase, phosphoenol pyruvate, and pyruvate kinase, leading to the formation of pyruvate. The reduction of pyruvate by equimolar amounts of NADH to...
lactate in the presence of lactate dehydrogenase was monitored spectrophotometrically at 365 nm. A stoichiometry of 1 mol of NADH oxidized per mol of CoA thioester formed was taken as evidence for ADP formation, and a stoichiometry of 1 mol of equivalent NADH was converted to 2 mol of NAD was taken as evidence for ATP formation. Cell extract precipitated at a 60% saturation of ammonium sulfate was used, since some interfering substances were present in the cell extract. Extracts of cells grown aerobically with BA, 4-OHPA, B, Phe, adipate, and anacardic acid (the PA-producing strain) were screened for these activities. The enzyme activity refers to micromoles of PACoA formed per minute per milligram of protein in the protein fraction precipitated at 60% ammonium sulfate.

Fractionation of the active proteins obtained from the affinity chromatography and hydroxyapatite chromatography. For separation and preliminary identification of the PA-CoA ligase activity, the fractions collected during this washing step were subjected to alkaline hydrolysis in KOH (pH 10 at 70°C for 30 min), and the liberated free acids were determined by HPLC. In other assays [C-14]PAnA was used and the formed labeled CoA thioester was identified by autoradiography after separation on thin-layer chromatography (TLC) plates. In vivo formation of PACoA. To demonstrate the formation of PACoA in whole A. evansi cells as a result of the activity of PA-CoA ligase, cell suspensions (2 ml, with an optical density at 578 nm of about 25) of PA- and Phe-grown cells were suspended in growth medium lacking a carbon source. These cells were fed with 0.5 mM PA plus 37 Kbp of [14]CoAP or 0.5 mM Phe plus 37 Kbp of [14]Phe and incubated at 37°C under aeration. At different time intervals (0 and 30 s and 1, 2, and 5 min) samples of 300 ml were withdrawn and centrifuged at 4°C for 5 min at 14,000 rpm. The cell pellet was washed once with ice-cold buffer lacking an aromatic substrate and extracted with hot acidic ethanol (pH 4, 80°C). The ethanolic extract was evaporated, and the resulting residue was dissolved in 30 ml of 0.05 M Tris-HCl and the fractions were collected directly by HPLC and used for determination of PAnA and PACoA contents.

Purification of PA-CoA ligase. PA-CoA ligase was purified from 20 g of cells. In the following chromatographic steps, the equilibration buffer used contained 10 mM Tris-HCl (pH 7.8), 2 mM MgCl2, 2 mM DTE, and 10% (w/v) glycerol. 200 ml of equilibration buffer. Fractions of 15 ml were collected. The PA-CoA ligase activity was detected in the fractions collected during this washing step. The rest of the contaminating proteins remained bound to the matrix and were washed off with 300 ml of equilibration buffer containing 500 mM KCl. The active fractions were pooled (118 ml) and diluted with the equilibration buffer (2 ml plus 2 volumes of buffer) to adjust the concentration of KCl to around 20 mM.

Anion-exchange chromatography on DEAE Bio-Gel A (Bio-Rad) (positive chromatography). The diluted protein sample was applied to another DEAE column (32-cm2 matrix) which had been equilibrated with 200 ml of equilibration buffer containing 20 mM KCl. The loaded column was washed with 100 ml of the same buffer at a flow rate of 0.2 ml min−1, and fractions of 10 ml were collected. The elution of PA-CoA ligase activity was achieved with a linear KCl gradient from 20 to 70 mM in 150 ml of equilibration buffer and then with 50 ml of buffer containing 70 mM KCl. The CoA ligase activity was eluted at the end of this gradient step by washing the column with 70 mM KCl.

Anion-exchange chromatography on Q-Sepharose. The active protein sample from the above-described step (59 ml) was applied directly at a flow rate of 2 ml min−1 to a fast protein liquid chromatography Q-Sepharose column (25 cm2; Pharmacia) which had been equilibrated with 150 ml of equilibration buffer containing 70 mM KCl. The loaded column was washed with 75 ml of the same buffer followed by 150 ml of a linear gradient from 70 to 150 mM KCl in 50 ml. The molecular mass of the native protein was determined by gel filtration chromatography on a calibrated Superdex 200 HR 10/30 column (Pharmacia). Half of the purified protein sample obtained from the preceding chromatographic step was precipitated with ammonium sulfate at a 60% saturation, and the resulting protein pellet was dissolved in 300 ml of equilibration buffer. The protein sample was applied to the column, which had been equilibrated with 5 bed volumes of the equilibration buffer at a flow rate of 0.5 ml min−1. Protein elution was monitored at an A 280 nm and fractions of 0.5 ml were collected and tested for activity. The molecular mass of the purified enzyme was estimated as described before (30), using protein molecular weight standards which contained catalase, aldolase, bovine serum albumin, ovalbumin, and chymotrypsinogen (molecular masses, 240, 158, 67, 43, and 25 kDa, respectively).

Cloning and DNA manipulations. Standard protocols were used for DNA cloning, transformation, amplification, and purification (6, 36). A-λ-ZAP Express gene library containing chromosomal DNA of A. evansi after Sau3A1 digestion was constructed as described in the ZAP Express cloning kit instruction manual (Stratagene). A 17-mer degenerate oligonucleotide (P0) was designed on the basis of the determined N-terminal amino acid sequence. Another four reverse 20-mer degenerate oligonucleotides (P1, P2, P3, and P4) were used and the formed labeled CoA thioester was identified by autoradiography after separation on thin-layer chromatography (TLC) plates. For separation and preliminary identification of the PA-CoA ligase activity, the fractions collected during this washing step were subjected to alkaline hydrolysis in KOH (pH 10 at 70°C for 30 min), and the liberated free acids were determined by HPLC. In other assays [C-14]PAnA was used and the formed labeled CoA thioester was identified by autoradiography after separation on thin-layer chromatography (TLC) plates.

Chemicals and biochemicals. Chemicals and biochemicals were from Gerbu (Gamburg, Germany), Boehringer (Mannheim, Germany), Sigma, and Fluka (Neu-Ulm, Germany). Radiolabeled [14]CoA and [14]Phe were from American Radiolabeled Chemicals/Biotechnology Chemicals (Cologne, Germany). PACoA was chemically synthesized according to established methods (18, 24).

DNA sequencing and computer analysis. Purification of plasmid DNA was performed according to a spin miniprep kit protocol (QiaGen). Sequencing of the DNA insert was carried out by J. Alt-Mörbe (Labor für DNA-Analytik, Freiburg, Germany). DNA and amino acid sequences were analyzed with the BLAST network service at the National Center for Biotechnology Information (Bethesda, Md.).

E. coli putative PA-CoA ligase genes were synthesized. Different PCR assays were performed with various combinations of P1, P2, P3, and P4 primers. A 450-bp PCR product was obtained with a combination of P1 and P2. This PCR product was labeled with [y-32P]ATP and was used as a probe to screen the constructed gene library. Five positive clones were obtained, and the resultant plasmids were maintained in E. coli XL-1 Blue/RI E.

DNA sequencing and computer analysis. Purification of plasmid DNA was performed according to a spin miniprep kit protocol (Qiagen). Sequencing of the DNA insert was carried out by J. Alt-Mörbe (Labor für DNA-Analytik, Freiburg, Germany). DNA and amino acid sequences were analyzed with the BLAST network service at the National Center for Biotechnology Information (Bethesda, Md.).

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from 2 to 80% methanol in 40 mM formic acid within 27 min. The retention times for PA and Phe were 33 and 15 min, respectively. (ii) For separation of PA and Phe a reverse-phase C_{18} column (Grom-Sil octadecyl silane-4HE, 5-μm particle size, 120 by 4 mm; Grom) was used. The mobil phase was a gradient of acetonitrile (2 to 40%) in 50 mM potassium phosphate buffer (pH 4.5) within 45 min. The retention times of PA and PACoA were 14 and 17 min, respectively. Radioactivity determination. Radioactive spots separated by TLC were scraped from the plates, and their radioactivities were determined by liquid scintillation counting. Radioactive peaks separated by HPLC were monitored by a flowthrough radioactivity detector with a solid scintillator cell. The radioactivities of these peaks were quantified by determination of peak area integration in comparison to that of a standard labeled compound.

Nucleotide sequence accession number. The sequence data reported in this article were submitted to the EMBL database (accession no. AF176259).

RESULTS

Growth characteristics and time course of PA-CoA ligase activity in aerobically growing cells. The aerobic growth of A. evansi on PA in chemically defined medium containing a 5 mM concentration of the aromatic substrate as the sole carbon source was studied. A. evansi consumed PA rapidly, with a maximal growth rate of 0.23 h^{-1} at 37°C. The molar growth yield was 56 g of dry cell mass formed per mol of PA consumed. The calculated specific substrate consumption rate of the culture was 114 nmol min^{-1} mg of protein^{-1}, assuming that 60% of the cell dry matter was protein (31).

The level of PA-CoA ligase activity in extracts (the protein fraction precipitated at 60% ammonium sulfate) of cells obtained during aerobic growth with PA was determined. The catalytic ability to activate PA was present early in the exponential growth phase and increased rapidly, reaching a maximal specific activity of 76 nmol min^{-1} mg of protein^{-1} within 6 h of incubation. This level of activity was maintained as long as PA was present in the medium; thereafter, about a 60% drop in activity was observed when PA was completely exhausted.

Induction pattern of PA-CoA ligase activity. To study the induction of PA-CoA ligase in cells grown with different aromatic or aliphatic substrates as the sole carbon source, extracts of cells grown aerobically with PA, 4-OHPA, Phe, B, acetate, and adipate and anaerobically with PA were screened for this activity (Table 1). The protein fraction (precipitated at 60% ammonium sulfate) of the cell extract centrifuged at 100,000×g was used, since the cell extracts of A. evansi have a high level of activity of endogenous NADPH oxidation which interferes with the detection of CoA ligase activity coupled to NADH oxidation. In addition, it turned out that the assay was inhibited by unknown compounds present in the soluble protein fraction.

When PA was the aromatic substrate to be tested, it was activated to its CoA thioester by extracts of PA- or Phe-grown cells at specific activities of 153 and 194 nmol min^{-1} mg of protein^{-1}, respectively. Much lower activities (about 11% or less) were measured in other cell extracts. In contrast, when 4-OHPA was the test substrate, much lower activities were measured (≤15% of those with PA) and they were nearly at the same rate in PA, Phe, and 4-OHPA cell extracts. High CoA ligase activities against B were detected in cell extracts aerobically grown with B and anaerobically grown with PA. These results clearly indicate that aerobic growth of A. evansi on PA or Phe and anaerobic growth on PA induces the synthesis of specific PA-CoA ligases. This activity was nearly or totally lost when cells were grown with other aromatic or aliphatic substrates. The trace activities toward 4-OHPA or B in extracts of PA- or Phe-grown cells may be due to the presence of other CoA ligases.

In vivo formation of PACoA. Cells incubated with labeled PA or Phe rapidly synthesized a labeled product which was detected very early after 30 s (Fig. 2). The retention time and the R_{f} value of this product matched exactly those of the authentic PACoA sample. The amount of this early product increased with time, and later on the product was consumed and other labeled products were formed which have not yet been identified. Alkaline hydrolysis of this product generated labeled PA. This result indicated that PACoA is the first true metabolite formed in cells growing with PA as the sole carbon source.

Purification of PA-CoA ligase. The purification of the PA-CoA ligase was achieved by a protocol which involved five chromatographic steps (Table 2). The recovered activity obtained after the first step (negative chromatography on DEAE-Sephrose) was considered 100% because of the difficulty of estimating accurately the activity in the soluble protein fraction. Although, the specific elution of the enzyme by its substrate from an affinity matrix (Reactive-Green 19) resulted in loss of two-thirds of the activity, a 15-fold purification was

![FIG. 2. In vivo formation of [14C]PACoA in A. evansi cells grown aerobically in the presence of [14C]PA (A) or [14C]phenylalanine (B). The reactions were stopped with 10% formic acid and separated by TLC followed by autoradiography. Lanes 1 to 4, samples taken at 0 and 30 s and 1 and 2 min in the presence of PA; lanes 5 to 8, samples taken at 30 s and 1, 2, and 5 min in the presence of phenylalanine. X indicates an unknown product.](http://jb.asm.org/ Downloaded from)
analyzed by TLC and identified by its Rf. With [14C]PA, the formation of labeled PACoA was also observed before and after alkaline hydrolysis to liberate free PA. The formed PACoA was identified by HPLC (Fig. 4A). This ratio indicates that the products are the oxidation of NADH.

Products and stoichiometry. The PA-CoA ligase reaction was dependent on PA, ATP, Mg\(^{2+}\), and CoA. The formation of PACoA was monitored spectrophotometrically at a wavelength of 365 nm in a reaction which allowed the rate of AMP or ADP formation to be determined by coupling the reaction via myokinase, pyruvate kinase, and lactate dehydrogenase to the oxidation of NADH. A stoichiometry of 2.0 mol of NADH oxidized per mol of PA added was observed. This ratio indicates that the products are PACoA and AMP plus pyrophosphate, rather than ADP plus phosphate. The formed PACoA was identified by HPLC (Fig. 4A) before and after alkaline hydrolysis to liberate free PA. With \(^{14}\)C]PA, the formation of labeled PACoA was also analyzed by TLC and identified by its Rf value by autoradiography (Fig. 4B). TLC and HPLC analyses of acid-stopped samples showed two labeled compounds which comigrated with authentic samples of PA and PACoA. After alkaline hydrolysis of the same samples, mainly one labeled compound which matched PA was detected by TLC (Fig. 4B) and HPLC. The radioactivity content of this compound was nearly equal to the total radioactivity present in the acid-stopped sample.

Substrate specificity. The substrate specificity of PA-CoA ligase was tested with the active fractions obtained with the hydroxyapatite column and also with the protein fractions showing the highest activities during the different chromatographic steps. The followings groups of molecules were tested: (i) aromatic acids and their ring-substituted (hydroxy-, carboxy-, amino-, and chloro-) derivatives, such as phenylbutyric, phenylpropionic, cinnamic, phenylacetic, mandelic, phenylglyoxylic, homophthalic, phthalic, and benzoic acids and phenylglycine and (ii) different aliphatic acids such as acetic, propionic, pyruvic, maleic, fumaric, succinic, bytural, acetooctic, and oxaloaotic acids. All active protein fractions obtained after the first purification step and the purified enzyme activated only phenylacetic acid, whereas all the other tested compounds were not used as substrates by PA-CoA ligase (≤2%).

Catalytic properties. The catalytic properties of the protein purified after the hydroxyapatite step were measured by the coupled enzyme assay described above (see Materials and Methods). The dependence of the activity on the pH was tested in Na-citrate (pHs 5 to 6), K-phosphate (pHs 6 to 8), and Tris-HCl (pHS 7 to 9) buffers, (100 mM each). The enzyme showed maximal activity at pHs 8 to 8.5, with a dramatic drop of activity (55%) at pH 9. Less than 10% activity was observed at pH 6, and half the maximal activity was measured at pH 7. ATP was the only nucleotide triphosphate accepted (100%), while the other tested nucleotides (CTP, GTP, and UTP) were not used (<2%). Also, N-acetylcysteamine (2 mM) could not substitute for CoA.

The apparent K_m values of the purified enzyme were determined for PA (10 μM to 5 mM), ATP (10 μM to 2 mM), and CoA (10 μM to 1 mM) at 37°C in assays containing 15 μg of protein. The reaction was started by adding various concentra-

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Total protein (mg)</th>
<th>Total enzyme activity (μmol min⁻¹ mg of protein⁻¹)</th>
<th>Sp act (μmol min⁻¹ mg of protein⁻¹)</th>
<th>Yield (%)</th>
<th>Level of purification (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soluble protein fraction (100,000 × g)</td>
<td>1,714</td>
<td>130.3</td>
<td>0.076</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>DEAE (negative chromatography)</td>
<td>463</td>
<td>191.2</td>
<td>0.413</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>DEAE (positive chromatography)</td>
<td>191</td>
<td>167.1</td>
<td>0.875</td>
<td>87.0</td>
<td>2</td>
</tr>
<tr>
<td>Q-Sepharose</td>
<td>67</td>
<td>131.3</td>
<td>1.960</td>
<td>69.0</td>
<td>5</td>
</tr>
<tr>
<td>Reactive-Green</td>
<td>1.6</td>
<td>46.9</td>
<td>29.31</td>
<td>24.5</td>
<td>71</td>
</tr>
<tr>
<td>Hydroxypatite</td>
<td>0.8</td>
<td>38.7</td>
<td>48.40</td>
<td>20.0</td>
<td>117</td>
</tr>
</tbody>
</table>

a Twenty grams of cells and 40 ml of buffer was used. The enzyme activities in the collected protein fractions were measured by the coupled enzyme assay.

b Enzyme activity is inhibited in crude cell extract and increases after the first chromatographic step. Therefore, values for yields and levels of purification (fold) were calculated in relation to the activity obtained after the first chromatographic step.

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tions of one substrate to an assay containing nonlimiting concentrations of the other cosubstrates. The $K_m$ values were determined from linear Lineweaver-Burk plots for each substrate. The enzyme showed high affinities towards its substrate and cosubstrates ($K_m$ values of 14, 60, and 45 mM for PA, ATP, and CoA, respectively). A turnover number (catalytic constant) of 40 s$^{-1}$ at 37°C was calculated from the molecular mass of the purified enzyme (50 kDa) and the maximal specific activity of 48 nmol min$^{-1}$ mg of protein$^{-1}$.

In presence of thiol group-modifying reagents such as N-ethylmaleimide and iodoacetamide (1 mM), no activity was detected. Severe inhibition of activity was observed in the presence of a 1 mM concentration of the divalent cations Zn$^{2+}$, Cu$^{2+}$, and Ni$^{2+}$ (≥80% inhibition). Also, a dramatic loss of activity (84%) was observed in the presence of NaF (2 mM), a reagent that inhibits some Mg$^{2+}$-requiring enzymes. The activity was absolutely dependent on the cocatalyst Mg$^{2+}$ (5 mM); Mn$^{2+}$ (5 mM) could partially replace Mg$^{2+}$, with 36% of the activity being recovered.

The enzyme was extremely labile, and the activity was totally lost within 48 h at 4°C. Glycerol was necessary for stabilizing the enzyme activity. At 10% glycerol (wt/vol), about 65% of the activity was recovered after 72 h at 4°C. The enzyme activity was more stable in 20% glycerol (80% recovery after 72 h at 4°C), but for practical reasons, the purification protocol was carried out in buffers containing only 10% glycerol. At −20°C in the presence of 10% glycerol, the enzyme activity was retained for 2 months without remarkable loss.

Cloning and sequencing of the gene coding for PA-CoA ligase. A 17-mer degenerated oligonucleotide primer was derived from the determined N-terminal amino acid sequence and was used in PCR assays against different reverse primers, which were deduced from conserved regions of the putative $E$. coli and $P$. putida PA-CoA ligases (see Materials and Methods). A 450-bp DNA fragment which showed similarity with other PA-CoA ligase genes was obtained. This PCR product was used to screen a λ-ZAP Express gene library (Stratagene) containing chromosomal DNA of $A$. evansii. Five positive clones were obtained and subsequently analyzed for the presence of the correct insert. Three of the clones contained 600 bp of the gene for PA-CoA ligase, while the other two clones

![Image](http://jb.asm.org/)

**TABLE 3. Biochemical and molecular characteristics of two PA-CoA ligase isoenzymes activating PA in $A$. evansii under aerobic and anaerobic growth conditions**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Aerobically</th>
<th>Anaerobically</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molecular mass (kDa)</td>
<td>48.752 (monomer)</td>
<td>49.376 (monomer)</td>
</tr>
<tr>
<td>N-terminal amino acid sequence</td>
<td>MPVKTPSPG</td>
<td>SARDGFAVP</td>
</tr>
<tr>
<td>pI</td>
<td>6.3</td>
<td>ND</td>
</tr>
<tr>
<td>Maximal sp act in growing cultures</td>
<td>76</td>
<td>48</td>
</tr>
<tr>
<td>($\text{nmol min}^{-1} \text{mg of protein}^{-1}$)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$K_m$ (µM) with:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PA</td>
<td>14</td>
<td>60</td>
</tr>
<tr>
<td>ATP</td>
<td>60</td>
<td>290</td>
</tr>
<tr>
<td>CoA</td>
<td>45</td>
<td>150</td>
</tr>
<tr>
<td>Turnover no. ($k_{cat}$ [s$^{-1}$])</td>
<td>40</td>
<td>22</td>
</tr>
<tr>
<td>Substrate specificity</td>
<td>Highly specific, PA only</td>
<td>Highly specific, PA only</td>
</tr>
<tr>
<td>Optimal pH</td>
<td>8 to 8.5</td>
<td>8.5</td>
</tr>
<tr>
<td>Induction growth conditions</td>
<td>Aerobic growth with PA or Phe</td>
<td>Anaerobic growth with PA or Phe plus nitrate</td>
</tr>
<tr>
<td>Enzyme stability</td>
<td>Requires glycerol</td>
<td>Requires glycerol</td>
</tr>
</tbody>
</table>

$^a$ The kinetic values for the two isoenzymes were determined under the same assay conditions.

$^b$ Data are from references 19, 30, and 39. ND, not determined.

$^c$ $k_{cat}$, catalytic constant.
carried the complete gene. The gene codes for 440 amino acids (1,320 bp) which correspond to a polypeptide of 48.75 kDa. This mass agrees well with the determined mass of the purified PA-CoA ligase (50 kDa by SDS-PAGE). The N-terminal amino acid sequence determined for the purified enzyme was identical with that deduced from the nucleotide sequence of PA-CoA ligase gene, thus confirming it as the gene product and showing that no processing of its N terminus occurs. A theoretical pI value of 6.26 was calculated from the deduced amino acid sequence of PA-CoA ligase, which agrees with the experimentally determined values (6.1 to 6.5) for the purified protein.

No potential open reading frames were found within 100 bp upstream of the 5′ end or within 200 bp downstream of the 3′ end of the gene.

**DISCUSSION**

CoA thioesterification of aromatic compounds is often necessary under anaerobic conditions to activate these compounds as a prerequisite to destabilize the inert aromatic ring prior to further transformations (20). The alternative widely distributed strategy followed under the aerobic conditions to destabilize the aromatic ring involves ring hydroxylation reactions which render the aromatic ring suitable for oxygenolytic ring fission.

Recently, an unusual new aerobic strategy that resembles the anaerobic degradation mechanism in CoA activation of the aromatic compounds prior to ring attack has been reported for some bacterial species (see the introduction).

The β-subclass proteobacterium *A. evansi* efficiently utilizes PA under both aerobic and anaerobic growth conditions. A
specific PA-CoA ligase, which has been purified (30), was found to be induced when cells were grown anaerobically with PA. The same activity was detected also in cells grown aerobically with PA. Hence, it was not clear whether this activity in cells grown aerobically and anaerobically with PA is due to the same enzyme or to another isoenzyme whose induction is restricted to the presence of PA under aerobic conditions. The induction of this activity under aerobic conditions was dependent on the presence of PA or Phe. The very low CoA-ligase activity detected with PA in 4-OHPA cell extract may be due to the presence of another nonspecific CoA ligase with a broad substrate spectrum or to the weak induction of PA-CoA ligase by the substrate analogue 4-OHPA. The substrate specificity of the purified enzyme indicated that the CoA ligase activities towards 4-OHPA and B detected in extracts of cells aerobically grown with PA are due to other CoA ligases. The following data indicate that the potential inducer molecule for the synthesis of the aerobic PA-CoA ligase in A. evansii may be PA or PACoA but not other aromatic substrates: (i) the detection of this activity very early in the exponential growth phase; (ii) the decrease in activity when PA was exhausted in the medium; (iii) the detectability of this activity also in Phe-grown cells, which most likely metabolize Phe via PA and PACoA; and (iv) the high substrate affinity and specificity of this enzyme. All these data in addition to the absence of hydroxylase activity with the free acid show that PACoA is the first true metabolite derived from PA during the aerobic metabolism of this acid.

The N-terminal amino acid sequences of the induced PA-CoA ligases under aerobic and anaerobic conditions in A. evansii were different (Table 3); hence, it was clear that PA is activated by two isoenzymes whose expression in this organism depends on the prevailing growth conditions. The two isoenzymes could also be differentiated by their kinetic properties (Table 3). The aerobically induced isoenzyme has a greater affinity for the aromatic substrate and the cosubstrates. This finding is consistent with an enzyme having a role in the PA aerobic degradation pathway. This enzyme initiates the activation of PA, facilitating the subsequent ring hydroxylation reactions, which, so far, are not easily demonstrated. The enzyme purified in this work is the second characterized CoA ligase activating PA under aerobic conditions. The substrate affinity of the A. evansii isoenzyme (14 μM for PA) is about a thousandfold higher than that reported for the first purified enzyme from P. putida (Km values for PA, ATP, and CoA were 16.5, 9.7, and 1.0 mM, respectively [29]). Thus, the sequences 239DIYGLSE245 and 305YRTRD309 (Fig. 5), which are conserved in all putative or proven PA-CoA ligases and match motifs II and III in acyl-adenylate-forming enzymes (16), probably do not contribute to the substrate-binding sites in PA-CoA ligases.

Although the reported PA-CoA ligases have different N-terminal amino acid sequences, they share many biochemical and catalytic features, such as (i) highly conserved amino acid motifs (Fig. 5), (ii) similar molecular masses of about 50 kDa, (iii) high substrate specificities, (iv) the requirement of Mg2+ for activity, (v) maximal catalytic activities in alkaline pHs (pHs 8 to 8.5), (vi) extreme lability and the requirement for glycerol for their stabilization, and (vii) inhibition of activity by divalent cations (Cu2+, Ni2+, and Zn2+). These data support the opinion that the specific induction of these enzymes in PA-degrading bacteria occurs when PA or other aromatic substrates metabolized via PA or PACoA (such as aromatic amino acids, lignin-related monooaromatic acids, aromatics with even-number carbon atoms of the side chain, styrene, phenylethanol, 2-phenylethylamine, and tyramine) serve as the sole carbon and energy sources.

A typical ribosome-binding site (AGGAG) is found 8 bases upstream of the potential ATG start codon of this gene. The gene product of A. evansii showed that it is closely related to other sequenced PA-CoA ligases. The derived amino acid sequence showed high identity to the corresponding putative PA-CoA ligase gene products of E. coli (PaaK, 64.6% (9), Pseudomonas sp. strain Y2 (PaaK, 64.7%) (44), and B. halodurans (open reading frame 12, 50%) (42); the PA-CoA ligase gene product of P. putida (PheA, 66.5%) (33); and the coenzyme F_{250} gene product of Methanobacterium thermoautotrophicum (MT1 161, 46%) (40). As shown by protein sequence alignments, a typical amino acid consensus sequence for an AMP binding motif (VX2SSGTTGKPTV) which is shared in other PA-CoA ligases (Fig. 5) was identified.

However, these sequence alignments have not allowed the identification of the consensus sequence essential for enzyme catalysis. Also, no sequence information data are available to explain the lability of these enzymes and their stabilization in the presence of glycerol.

The PA-CoA ligase gene of A. evansii was designated paaK according to the abbreviations for the putative genes probably involved in PA catabolism in E. coli K-12.

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