Purification and Characterization of Benzoate-Coenzyme A Ligase and 2-Aminobenzoate-Coenzyme A Ligases from a Denitrifying Pseudomonas sp.

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The enzymes catalyzing the formation of coenzyme A (CoA) thioesters of benzoate and 2-aminobenzoate were studied in a denitrifying Pseudomonas sp. anaerobically grown with these aromatic acids and nitrate as sole carbon and energy sources. Three different rather specific aromatic acyl-CoA ligases, E₁, E₂, and E₃, were found which catalyze the formation of CoA thioesters of benzoate, fluorobenzoates, and 2-aminobenzoate. ATP is cleaved into AMP and pyrophosphate. The enzymes were purified, their N-terminal amino acid sequences were determined, and their catalytic and molecular properties were studied. Cells anaerobically grown on benzoate and nitrate contain one CoA ligase (AMP forming) for benzoic acid (E₁). It is a homodimer of M₁ 120,000 which prefers benzoate as a substrate but shows some activity also with 2-aminobenzoate and fluorobenzoates, although with lower Kₛ. Cells anaerobically grown on 2-aminobenzoate and nitrate contain three different CoA ligases for aromatic acids. The first one is identical with benzoate-CoA ligase (E₂). The second enzyme is a 2-aminobenzoate-CoA ligase (E₃). It is a monomer of M₁ 60,000 which prefers 2-aminobenzoate but also activates benzoate, fluorobenzoates and, less effectively, 2-methylbenzoate, with lower affinities to the latter substrates. The enzymes E₁ and E₃ have similar activity levels; a third minor CoA ligase activity is due to a different 2-aminobenzoate-CoA ligase. This enzyme (E₃') is a monomer of M₁ 65,000 which is identical to an isoenzyme 2-aminobenzoate-CoA ligase, operating in a new plasmid-encoded aerobic 2-aminobenzoate pathway (U. Altenschmidt, C. Eckerskorn, and G. Fuchs, Eur. J. Biochem. 194:647-653, 1990); apparently, it is not completely repressed under anaerobic conditions and therefore also is induced to a small extent by 2-aminobenzoate under anaerobic growth conditions.

Aromatic compounds are metabolized by microorganisms by two fundamentally different methods. Under aerobic conditions, aromatic compounds are transformed by monooxygenases and dioxygenases into a few central intermediates such as catechol, protocatechuate, and gentisate. These compounds are suitable for an oxidative chemical attack. Accordingly, the aromatic ring structures are cleaved enzymatically by dioxygenases (for a review, see reference 11).

Under anaerobic conditions, aromatic compounds have to be transformed by other means than by oxygenases. Figure 1 gives an outline of the initial reactions in the anaerobic metabolism of some aromatic compounds leading to the most important central intermediate, benzoyl-coenzyme A (CoA), as studied in denitrifying Pseudomonas species. These reactions have three functions. The first function is to activate chemically inert compounds such as phenol or toluene and others; CoA thioester formation of aromatic acids is one notable form of activation. CoA ligases for the aromatic acids benzoate (10) and 4-hydroxybenzoate (9a) from anaerobically grown Rhodopseudomonas palustris and for phenylacetate (22) from aerobically grown Pseudomonas putida have been purified before. The second function is to reduce the enormous variety of natural and synthetic aromatic compounds, channeling them into a few central intermediates. The third function is to direct to those intermediates compounds such as benzoyl-CoA (rather than benzoate), resorcinol, and phloroglucinol which are suitable for a reductive attack of the aromatic nucleus. In keeping with this, these central aromatic compounds appear to be attacked enzymatically by reductases, and the resulting alicyclic compounds have been shown or postulated to become hydrolytically cleaved (7, 9, 14, 17-19).

The present work aimed at studying the initial reactions and enzymes in the anaerobic metabolism of benzoate and 2-aminobenzoate. These aromatic acids are of biological importance and in addition are formed secondarily from a variety of aromatic precursors by microbial activity (13, 16). The bacterium studied, Pseudomonas strain KB 740™ (3), was anaerobically grown with nitrate as an electron acceptor and benzoate or 2-aminobenzoate as the sole source of cell carbon and electrons. Different CoA ligases for aromatic acids (acyl-CoA synthetases) such as benzoate, 2-aminobenzoate, 4-hydroxybenzoate, and (4-hydroxy)phenylacetate have been detected in this bacterium when anaerobically grown on the respective acids (12, 28, 30, 35). We have recently disclosed a new aerobic, plasmid-encoded pathway of 2-aminobenzoate metabolism in the same organism which, unexpectedly, also proceeds via 2-aminobenzyol-CoA but is under aerobic control; therefore, it involves a 2-aminobenzoate-CoA ligase even under aerobic growth conditions (1a).

Here, we report on the demonstration and purification of three different CoA ligases, those for anaerobic benzoate and 2-aminobenzoate metabolism and for aerobic 2-aminobenzoate metabolism. The expressions of these enzyme activities are controlled by different means. The knowledge of the N-terminal amino acid sequences of these enzymes will enable us to study some molecular biological aspects of

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FIG. 1. Outline of reactions and intermediates involved in the initial steps of the anaerobic metabolism of various aromatic compounds via benzoyl-CoA as central aromatic intermediate. The reactions and enzymes and their regulation patterns have been studied in the denitrifying *Pseudomonas* strains KB 740' and KB 172. They involve CoA thioester formation (references 5 and 35 and this paper), carboxylations (20, 31), oxidative decarboxylations (5, 30), anaerobic hydroxylations with water acting as the source of oxygen (dehydrogenases) (references 1b and 30 and unpublished data), as well as reductive eliminations of aromatic hydroxyl (12) and possibly of amino substituents.

the metabolism of aromatic acids under anaerobic and aerobic conditions.

**MATERIALS AND METHODS**

**Materials.** Chemicals and medium components were obtained from Merck (Darmstadt, Germany), Roth (Karlsruhe, Germany), Difco (Hamburg, Germany), Fluka (Neu-Ulm, Germany), or Sigma (Heidelberg, Germany). Materials for column chromatography were obtained from Pharmacia (Freiburg, Germany), Fluka, Bio-Rad (Munich, Germany), and Sigma. Fast protein liquid chromatography (FPLC) equipment was from Pharmacia. Biochemicals were obtained from Boehringer (Mannheim, Germany), Fluka, Pharmacia, or Sigma. Gases were from Linde (Höllriegelskreut, Germany). *Pseudomonas* strain KB 740' was a kind gift from Konstantin Braun.

**Growth of bacteria.** *Pseudomonas* strain KB 740' was grown aerobically or anaerobically (plus nitrate), with benzoate or 2-aminobenzoate as sole carbon and energy sources, essentially as described previously (3). Growth determination, growth yield determination, cell harvest, and storage were described previously (35).

**Assays of CoA ligase activity.** The determinations of benzoate-CoA ligase activity and of 2-aminobenzoate-CoA ligase activity, as well as the determination of the reaction stoichiometry, were accomplished with spectrophotometric assays as described previously (35).

**Determinations.** Benzoate and 2-aminobenzoate as well as their CoA thioesters were separated by high-pressure liquid chromatography (HPLC) (34). 2-Aminobenzoyl-CoA was characterized by UV and nuclear magnetic resonance spectroscopy as described previously (4), and benzoyl-CoA was characterized by UV spectroscopy (32). CoA thioesters were hydrolyzed (6).

**Protein determination.** Protein was determined by the method of Bradford (2), using crystalline bovine serum albumin as the standard.

**SDS-PAGE.** Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; 12.5% polyacrylamide) was carried out at 4°C with the discontinuous buffer system of Laemmli (21). Polypeptides were visualized by silver staining (24).

**Preparation of cell extract.** All subsequent procedures were carried out at 0 to 4°C. About 10 g (wet weight) of harvested cells was suspended in 20 ml of a 100 mM tris(hydroxymethyl)aminomethane-HCl (Tris-HCl) buffer (pH 7.8) containing 2 mM dithioerythritol (DTE) and 2 mM MgCl₂ and passed twice through a French pressure cell (American Instruments Company) at a pressure of 137 MPa. Unbroken cells and cell debris were removed by ultracentrifugation at 100,000 × g for 1 h. The resulting supernatant (about 26 ml) was termed the soluble protein fraction.

**Purification of CoA ligases.** The purification scheme is summarized in Fig. 2. All steps were carried out at 4°C.

(i) **Ammonium sulfate precipitation and desalting with a Sephadex-G 25 column.** The soluble protein fraction was treated with ammonium sulfate to 30% of saturation and stirred for 30 min. After centrifugation at 12,000 × g for 15 min, the supernatant containing CoA ligase activity was passed through a Sephadex-G 25 column (diameter, 3.2 cm; volume, 230 ml) and equilibrated with Tris-HCl buffer (10

FIG. 2. Scheme of purification protocols for the three CoA ligases (AMP forming) E₁, E₂, and E₃ acting on the aromatic acids benzoate and 2-aminobenzoate.
mM Tris-HCl [pH 7.8], 2 mM DTE, 2 mM MgCl₂) to eliminate ammonium sulfate.

(ii) DEAE-cellulose chromatography. The fractions with ligase activity (110 ml) were applied at a flow rate of 2 ml min⁻¹ to a DEAE-Sephascel column (diameter, 3.2 cm; volume, 64 ml) that had been equilibrated with Tris-HCl buffer (10 mM Tris-HCl [pH 7.8], 2 mM DTE, 2 mM MgCl₂, 50 mM KCl). The column was washed with 150 ml of the same buffer and eluted with a stepwise KCl gradient (step 1, 150 ml of 100 mM KCl; step 2, 150 ml of 150 mM KCl). Fractions of 10 ml were collected. The 2-aminobenzoate-CoA ligase (E₁) was eluted with 50 mM KCl.

(iii) Hydroxyapatite chromatography. The fractions (100 ml) with 2-aminobenzoate-CoA ligase activity were applied directly to a hydroxyapatite column (diameter, 2.4 cm; volume, 25 ml) at a flow rate of 1 ml min⁻¹. The column was equilibrated beforehand with a potassium phosphate buffer (5 mM, pH 7.8) containing 2 mM DTE and 2 mM MgCl₂. The column was washed with 100 ml of the same buffer. One 2-aminobenzoate-CoA ligase (E₁) was eluted with buffer. A second 2-aminobenzoate-CoA ligase (E₂) was eluted in a 200-ml linear potassium phosphate gradient (5 to 20 mM). Fractions of 4 ml were collected.

(iv) Q-Sepharose chromatography. The fractions containing benzoate-CoA ligase (E₁) from the DEAE chromatography and the two 2-aminobenzoate-CoA ligases (E₂ and E₃) from the hydroxyapatite chromatography were applied to a Q-Sepharose column (diameter, 1.6 cm; volume, 20 ml) at a flow rate of 3 ml min⁻¹. To elute the benzoate-CoA ligase, the Q-Sepharose column was equilibrated and washed with a Tris-HCl buffer (10 mM Tris-HCl [pH 7.8], 2 mM DTE, 2 mM MgCl₂, 230 mM KCl). The ligase was eluted in a 200-ml linear KCl gradient (230 to 340 mM KCl). To elute both 2-aminobenzoate-CoA ligases, the column was equilibrated and washed with a Tris-HCl buffer (10 mM Tris-HCl [pH 7.8], 2 mM DTE, 2 mM MgCl₂, 125 mM KCl). The enzymes were eluted in a 200-ml linear KCl gradient (125 to 175 mM KCl). Three-milliliter fractions containing ligase activity were collected and pooled.

(v) Affinity chromatography. Q-Sepharose-purified CoA ligases were applied at a flow rate of 0.5 ml min⁻¹ to a “reactive green” cross-linked agarose column (diameter, 4.4 cm; volume, 23 ml) equilibrated with a buffer of Tris-HCl (pH 7.8) containing 2 mM DTE and 2 mM MgCl₂. The column was washed with the same buffer. Benzoate-CoA ligase (E₁) was eluted with 0.5 M KCl; one of the 2-aminobenzoate-CoA ligases (E₂) was eluted with 1 M KCl, and the second 2-aminobenzoate-CoA ligase (E₃) was eluted with 10 mM ATP. Fractions of 2 ml were collected.

(vi) Determination of the native molecular weight by gel filtration. An FPLC “Superdex 200” column (diameter, 1.6 cm; volume, 112 ml) was equilibrated with a 10 mM Tris-HCl buffer (pH 7.8) containing 2 mM DTE and 2 mM MgCl₂. Fractions (5 ml) containing ligase activity were applied to the column. The column was run at a flow rate of 0.8 ml min⁻¹, and 1-ml fractions were assayed for enzyme activity. The column was calibrated with the following Mₕ markers: ferritin, 440,000; catalase, 232,000; bovine serum albumin, 67,000; ovalbumin, 43,000.

Determination of N-terminal amino acid sequence. For determination of the N-terminal amino acid sequence, the CoA ligases were separated on an SDS-polyacrylamide gel. The proteins were stained with Coomassie blue and transferred to a siliconized glass fiber foil (Clossybond; Biometra, Göttingen, Germany), and the protein bands were cut off.

The sequences were determined in an automated gas-phase sequencer (model 470 A; Applied BioSystems, Weiterstadt, Germany) based on Edman degradation. The phenylthiodyantoin derivatives of the released amino acids were determined by C₄ reversed-phase HPLC (8).

RESULTS

Enzyme activities in cell extracts. Cell extracts (100,000 x g supernatant) of Pseudomonas strain KB 740 grown anaerobically with benzaldehyde or 2-aminobenzoate plus substrate as sole carbon and energy sources were tested for ATP-dependent CoA ligase activities acting on the aromatic acids supplied as the substrates. Cells from both cultures contained CoA ligase activity for benzaldehyde and 2-aminobenzoate. The highest amount of 2-aminobenzoate-CoA ligase activity (0.14 μmol of 2-aminobenzoyl-CoA formed min⁻¹ mg⁻¹ protein⁻¹) was obtained from 2-aminobenzoate-grown cells grown in the middle of the exponential growth phase (A₅₇₈ = 0.6); similarly, the highest benzaldehyde-CoA ligase activity was in benzaldehyde-grown cells from the logarithmic growth phase (0.24 μmol of benzoyl-CoA formed min⁻¹ mg⁻¹). The activity decreased to one-fourth in the stationary growth phase (0.03 to 0.96 μmol min⁻¹ mg⁻¹). Hereafter, one enzyme unit (U) refers to 1 μmol of acyl-CoA formed min⁻¹. It has to be taken into account that the benzaldehyde- and 2-aminobenzoate-CoA ligase activities were due to three different enzymes (see below). From these data alone, the specific activity of the individual enzymes in cell extracts cannot be estimated.

Purification of benzoate-CoA ligase (E₁) and 2-aminobenzoate-CoA ligases (E₂ and E₃). Three different soluble aromatic acid-CoA ligases were present in cells anaerobically grown on 2-aminobenzoate and one was present in cells anaerobically grown on benzaldehyde. Cells aerobically grown on the aromatic acids also contained CoA ligases for the respective substrates. This will not be followed up here. The procedure described above (Fig. 2) resulted in a 640-fold purification of the benzoate-CoA ligase (E₁), <1,430-fold purification of one 2-aminobenzoate-CoA ligase (E₂), and >120-fold purification of a second 2-aminobenzoate-CoA ligase (E₃) when cells aerobically grown on 2-aminobenzoate were analyzed (for purification factor, see Discussion) (Table 1). E₂ and E₃ could only be separated during the later steps of the purification procedure. The enzyme E₁ was also purified from cells aerobically grown on 2-aminobenzoate (>550-fold purification); these data and the localization, cloning, and sequencing of the gene will be presented elsewhere. Fifty-nine percent of total E₁, 87% of total E₂, and 10% of E₃ were recovered. The highest content of enzyme activity was found in fractions after 30% ammonium sulfate treatment, because the ligases are inhibited in cell extracts by an unknown component which is lost during the purification (35). Final specific activities in the eluate from the affinity chromatography column ranged from 152 U per mg of protein for E₁ and 169 U per mg for E₂ to 16.5 U per mg for E₃. During the exploration stage of the purification, a number of affinity chromatography matrices were tested. Except for reactive green-agarose, none of the matrices were able to bind or to release the enzymes totally. The enzymes were found to elute with 1 M KCl (E₁), 0.5 M KCl (E₂), or 10 mM ATP (E₃). The kinetic properties of the enzymes are summarized in Table 2.

Nature of the products and stoichiometry of the reactions. The three enzymes were absolutely dependent on the aromatic acids, ATP, Mg²⁺, and CoA. The products of the
TABLE 1. Purification protocol for the three CoA ligases E₁, E₂, and E₃ acting on the aromatic acids benzoate and 2-aminobenzoate

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Vol (ml)</th>
<th>Total enzyme activity (U)</th>
<th>Protein concn (mg/ml)</th>
<th>Total protein (mg)</th>
<th>Sp act (U/mg of protein)</th>
<th>Yield of recovery (%)</th>
<th>Purification (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Purification of E₁</td>
<td>100,000 × g supernatant</td>
<td>E₁</td>
<td>26</td>
<td>150</td>
<td>24</td>
<td>624</td>
<td>0.236</td>
</tr>
<tr>
<td></td>
<td></td>
<td>E₁ + E₃</td>
<td>26</td>
<td>85</td>
<td>24</td>
<td>624</td>
<td>0.137</td>
</tr>
<tr>
<td></td>
<td>Ammonium sulfate precipitate</td>
<td>E₁</td>
<td>35</td>
<td>250</td>
<td>11.5</td>
<td>402</td>
<td>0.614</td>
</tr>
<tr>
<td></td>
<td></td>
<td>E₁ + E₃</td>
<td>37</td>
<td>216</td>
<td>11.5</td>
<td>425</td>
<td>0.505</td>
</tr>
<tr>
<td></td>
<td>Sephadex G-25</td>
<td>E₁</td>
<td>70</td>
<td>242</td>
<td>5.8</td>
<td>406</td>
<td>0.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>E₁ + E₃</td>
<td>70</td>
<td>190</td>
<td>5.8</td>
<td>406</td>
<td>0.47</td>
</tr>
<tr>
<td></td>
<td>DEAE-cellulose</td>
<td>E₁</td>
<td>60</td>
<td>246</td>
<td>1</td>
<td>60</td>
<td>4.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>E₁ + E₃</td>
<td>100</td>
<td>210</td>
<td>0.3</td>
<td>30</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Q-Sepharose, E₁</td>
<td>50</td>
<td>147</td>
<td>0.43</td>
<td>21.5</td>
<td>7.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reactive green-agarose, E₁</td>
<td>20</td>
<td>145</td>
<td>0.048</td>
<td>0.96</td>
<td>151.7</td>
</tr>
<tr>
<td>2. Purification of E₂</td>
<td>Hydroxyapatite, E₂</td>
<td>180</td>
<td>160</td>
<td>0.2</td>
<td>36</td>
<td>4.4</td>
<td>78</td>
</tr>
<tr>
<td></td>
<td>Q-Sepharose, E₂</td>
<td>20</td>
<td>207</td>
<td>0.27</td>
<td>5.4</td>
<td>42.1</td>
<td>98</td>
</tr>
<tr>
<td></td>
<td>Reactive green-agarose, E₂</td>
<td>15</td>
<td>190</td>
<td>0.072</td>
<td>1.08</td>
<td>169.2</td>
<td>87</td>
</tr>
<tr>
<td>3. Purification of E₃</td>
<td>Hydroxyapatite, E₃</td>
<td>30</td>
<td>22</td>
<td>0.1</td>
<td>3</td>
<td>7.2</td>
<td>10.7</td>
</tr>
<tr>
<td></td>
<td>Q-Sepharose, E₃</td>
<td>20</td>
<td>22</td>
<td>0.13</td>
<td>2.4</td>
<td>9.1</td>
<td>10.4</td>
</tr>
<tr>
<td></td>
<td>Reactive green-agarose, E₃</td>
<td>30</td>
<td>21</td>
<td>0.044</td>
<td>1.32</td>
<td>16.5</td>
<td>10</td>
</tr>
</tbody>
</table>

* The yield and purification factor for E₂ and E₃ cannot be given precisely. They refer to E₂ plus E₃ activity. If it is taken into account that E₃ activity was approximately 10% of E₂ activity, the values for E₂ will be up to 10 times higher and the values for E₃ will be ≥10% lower. Cells anaerobically grown on 2-aminobenzoate were used for purification of the enzymes. Similar specific activities of the purified proteins E₁ and E₂ were obtained when cells anaerobically grown on benzoate (E₁) or aerobically grown on 2-aminobenzoate (E₂) were used.

* E₁ was measured with benzoate as the substrate by using the spectrophotometric assay described in Materials and Methods.

* E₂ and E₃ were measured with 2-aminobenzoate as the substrate by using the spectrophotometric assay described in Materials and Methods.

reactions were the CoA thioesters of the respective acids and were characterized as follows: by UV spectroscopic characterization after HPLC purification, by nuclear magnetic resonance spectroscopy of 2-aminobenzyol-CoA, by cochromatography with authentic benzyol-CoA and 2-aminobenzyol-CoA on HPLC, and by detection of the free acids benzoate and 2-aminobenzoate by HPLC upon alkaline hydrolysis of the assay mixture after the assay had run to completion. The product of ATP hydrolysis was AMP rather than ADP in all cases. One mol of AMP was formed per mol of added ATP when the cosubstrates benzoate and CoA and the cocatalyst Mg²⁺ were present in excess; 1 mol of AMP was formed per mol of added benzoate when ATP, Mg²⁺, and CoA were present in excess (Fig. 3).

Substrate specificity. All three enzymes acted on benzoate, 2-aminobenzoate, and the three fluorobenzoate isomers; the specific rates with the different aromatic acids differed only by a factor of two to four. However, the differences in their substrate affinities, as indicated by the apparent Kₘ values (Table 2) (see below) were more pronounced. The kinetic and regulatory properties suggest that E₁ is a benzoate-CoA ligase and E₂ and E₃ are 2-aminobenzoate-CoA ligases. All three ligases used ATP preferentially; no other nucleotide triphosphate (UTP, GTP) was used. ADP and AMP were completely ineffective.

Acetate, cyclohexane carboxylate, and a selection of aromatic acids were tested as potential substrates (Table 3). Benzoate-CoA ligase (E₁) activated only benzoate and analogs with close steric resemblances, such as 2-fluorobenzoate and 4-fluorobenzoate. With 3-fluorobenzoate and 2-aminobenzoate, even less activity was observed. The 2-aminobenzoate-CoA ligases (E₂ and E₃) had very similar substrate specificities. Benzoate, 2-fluorobenzoate, and 4-fluorobenzoate were activated with the same reaction rate as 2-aminobenzoate. With 3-fluorobenzoate and 2-methylbenzoate, there was half and less than one-fifth, respectively, of the activity found with 2-aminobenzoate. The other substrates were not activated (≤1%).

Physicochemical properties of the ligases. In SDS-polyacrylamide gels (Fig. 4), the purified enzymes migrated as

TABLE 2. Catalytic properties of the CoA ligases E₁, E₂, and E₃ acting on the aromatic acids benzoate and 2-aminobenzoate

<table>
<thead>
<tr>
<th>CoA ligase</th>
<th>Turnover numbera (substrate)</th>
<th>Kₘ (μM)</th>
<th>pH optimum</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Benzoate</td>
<td>2-Aminobenzoate</td>
<td>2-Fluorobenzoate</td>
</tr>
<tr>
<td>E₁</td>
<td>26,000 (benzoate)</td>
<td>11</td>
<td>250</td>
</tr>
<tr>
<td>E₂</td>
<td>16,000 (2-aminobenzoate)</td>
<td>35</td>
<td>13</td>
</tr>
<tr>
<td>E₃</td>
<td>1,300 (2-aminobenzoate)</td>
<td>75</td>
<td>15</td>
</tr>
</tbody>
</table>

* The standard assay described in Materials and Methods was used.

a k₉₅ in min⁻¹ at 28°C.

b Determined with benzoate as cosubstrate.

c Determined with 2-aminobenzoate as cosubstrate.
The formation of AMP was indirectly measured by coupling the reaction to myokinase, pyruvate kinase, and lactate dehydrogenase reaction.

single bands. The calculated molecular weights were 55,000 for E₁, 60,000 for E₂, and 65,000 for E₃. The apparent molecular weights (Fig. 5) determined by gel filtration chromatography were 120,000 for E₁, 60,000 for E₂, and 65,000 for E₃. Hence, E₁ is a homodimer, whereas E₂ and E₃ are active as monomers. The spectra of the purified enzymes exhibited no significant absorption above 300 nm.

**N-terminal amino acid sequence of the enzymes and differential expression of the aromatic acid-CoA ligase activities.**

The N-terminal amino acid sequences of the enzymes were determined; they are shown in Table 4. The N-terminal amino acid sequence of benzoate-CoA ligase (E₁) was identical irrespective of whether the protein was obtained from cells anaerobically grown on benzoate or 2-aminobenzoate.

**TABLE 3. Substrate specificity of the three CoA ligases E₁, E₂, and E₃ acting on the aromatic acids benzoate and 2-aminobenzoate.**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>E₁</th>
<th>E₂</th>
<th>E₃</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benzoate</td>
<td>100</td>
<td>100</td>
<td>91</td>
</tr>
<tr>
<td>2-Aminobenzoate</td>
<td>28</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Cyclohexanecarboxylate</td>
<td>≤2</td>
<td>≤3</td>
<td>≤5</td>
</tr>
<tr>
<td>3-Aminobenzoate</td>
<td>≤3</td>
<td>≤4</td>
<td>≤5</td>
</tr>
<tr>
<td>2-Fluorobenzoate</td>
<td>120</td>
<td>110</td>
<td>100</td>
</tr>
<tr>
<td>3-Fluorobenzoate</td>
<td>76</td>
<td>56</td>
<td>50</td>
</tr>
<tr>
<td>4-Fluorobenzoate</td>
<td>100</td>
<td>100</td>
<td>90</td>
</tr>
<tr>
<td>2-Methylbenzoate</td>
<td>&lt;1</td>
<td>20</td>
<td>9</td>
</tr>
</tbody>
</table>

TABLE 3. Substrate specificity of the three CoA ligases E₁, E₂, and E₃ acting on the aromatic acids benzoate and 2-aminobenzoate.

It differed from the sequences of the two 2-aminobenzoate-CoA ligases (E₂ and E₃) which, surprisingly, appear to be almost identical, although the two proteins migrated at slightly different rates on the denaturing SDS-polyacrylamide gel. In addition, the expressions of E₂ and E₃ apparently were differently regulated in that the level of E₂ was low or not detectable under aerobic conditions, whereas E₃ was expressed at an approximately 10-fold-higher level aerobically than anaerobically.

**pH dependence.**

The rates of benzoyl-CoA and 2-aminobenzoyl-CoA formation at different pH values of Tris-HCl buffer were measured. The benzoate-CoA ligase (E₁) had a broad pH optimum between 8.5 and 9.2, with half-maximal activity at pH 7.5 and 9.9. pH optima of 9.3 for E₂, with half-maximal activity at pH 8.1 and 9.9, and of 8.5 for E₃, with half maximal activity at pH 7.4 and 9.3, were observed.

**Kinetics.**

The values of the Michaelis-Menten saturation kinetics and the turnover rates for benzoate, 2-aminobenzoate, ATP, and CoASH were obtained (Table 2). The apparent kinetic constants for the purified enzymes were determined from linear Lineweaver-Burk plots. The apparent Kₘ values were as follows: for the physiological aromatic acids, 10 to 15 μM; for ATP, 40 to 130 μM; for CoASH, 20 to 100 μM. Benzoyl-CoA and 2-aminobenzoate were the aromatic acids with the lowest apparent Kₘ values for E₁ and for E₂ and E₃, respectively. The turnover numbers of E₁ and E₂ were similar (in the order of 10⁴ min⁻¹), whereas E₃ had a kₗ in the order of 10³ min⁻¹.

**Stability and activity inhibition.**

The purified enzymes were not sensitive to oxygen. The enzymes could be stored frozen in liquid nitrogen for 3 months without remarkable loss of activity. When stored at 4°C at a protein concentration of 50 μg ml⁻¹ in Tris-HCl buffer, pH 7.8, half of the activity was lost in 4 days. Several agents that react with thiol groups [5,5'-dithiobis(2-nitrobenzoic acid) and p-chloromercuribenzoic acid] were tested at a concentration of 1 mM. p-Chloromercuribenzoic acid inhibited the benzoate-CoA
ligase (E₁) and the 2-aminobenzoate-CoA ligase (E₃) virtually completely but had no effect on 2-aminobenzoate-CoA ligase (E₂). E₁ and E₂ were totally inhibited by 5,5'-dithiobis-(2-nitrobenzoic acid), whereas E₃ was not inhibited. These results suggest that the presence of SH groups which are accessible to different degrees to these inhibitors are essential for the catalytic activity.

Inhibition effects of several univalent (1 mM) cations (K⁺, Na⁺, Li⁺, and Rb⁺) on the ligase activities were not detectable. Some divalent cations (1 mM) (Zn²⁺ and Cu²⁺) did cause strong inhibition; Cu²⁺ totally inhibited all enzymes, probably because of reaction with SH groups. Zn²⁺ inhibited E₁ strongly, whereas E₂ and E₃ were less sensitive. No inhibition was observed with Mn²⁺ ions. Mg²⁺ ions could be fully replaced by Mn²⁺ ions (5 mM) in the test. Detergents (1 mM) also affected the ligase activity. SDS inactivated the enzyme. Other molecules such as Tween 100 and N-octylglucoside inhibited the activities to a much lesser extent.

**DISCUSSION**

In the denitrifying *Pseudomonas* strain KB 740⁻ and in strain K 172 (5), at least six different aromatic acid-CoA ligases are present (30, 35), three of which have been purified in this investigation. E₁ is induced anaerobically, probably by benzoate; E₂ is induced anaerobically by 2-aminobenzoate; and E₃ is induced aerobically by 2-aminobenzoate. Enzymes which play a role in anaerobic metabolism are benzoate-CoA ligase E₁ (previously referred to as synthetase 1 [35]), 2-aminobenzoate-CoA ligase E₂ (previously referred to as synthetase 2 [35]), 4-hydroxybenzoate-CoA ligase, and phenylacetate-CoA ligase. In aerobic cells, a benzoate-CoA ligase of unknown function which may be slightly different from E₁ (unpublished results) and a 2-aminobenzoate-CoA ligase (1a) were found. E₂ is identical with the aerobic 2-aminoenzoate-CoA ligase isozyme and is probably coded on an 8.1-kbp plasmid which also carries the gene for 2-aminoenzyol-CoA monooxygenase/reductase, a key enzyme of this aerobic pathway (1, 1a). The total E₁ synthetase activity of cells grown anaerobically with 2-aminobenzoate was only 10% of the total 2-aminobenzoate-CoA ligase activity of cells grown aerobically with 2-aminobenzoate. This shows that the two 2-aminobenzoate-CoA ligases E₂ and E₃ are differently regulated. E₂ and E₃ differ also with respect to other properties; e.g., they exhibit different chromatographic behavior, turnover numbers, and molecular weights. In spite of these differences, E₁ and E₂ have similar Kₘ values, pH optima, and substrate specificities, and the N-terminal amino acid sequences were identical except for one amino acid position. Since these two enzymes could not be differentiated by simple activity measurement, the yield and purification factors given are only approximate values. The relation of the two enzymes to each other and the localization of the two genes remain to be studied.

In several bacteria able to be grown on aromatic compounds in the absence of molecular oxygen, similar enzymes were reported; they were proposed to be involved in the anaerobic metabolism of the following compounds (in parentheses): benzoate-CoA ligase (benzoate, benzoaldehyde, benzyl alcohol, and toluene) (1b, 5, 10, 15, 17, 25, 28, 35), 4-hydroxybenzoate-CoA ligase (phenol, p-cresol, and 4-hydroxybenzoate) (5, 9a, 12, 15, 23, 27, 35), 3-methylbenzoate-CoA ligase (3-methylbenzoate) (27), 4-hydroxy-3-methylbenzoate-CoA ligase (4-hydroxy-3-methylbenzoate, o-cresol, and 2,4-dimethylphenol) (27), 4-aminobenzoate-CoA ligase (aniline and 4-aminobenzoate) (29), phenylacetate-CoA ligase (phenylacetate and 4-hydroxyphenylacetate) (5, 30), phthalate-CoA ligase (o-, m-, and p-phthalate) (25), and 2-hydroxybenzoate-CoA ligase (salicylate) (15, 25). The list is certainly not complete. Of these enzymes, only benzoeote-CoA ligase and 4-hydroxybenzene-CoA ligase from *R. palustris* (9a, 10) have been purified. Furthermore, phenylpropanic acids and compounds transformed to C₆-C₃ compounds (33) may be metabolized via their CoA thioesters through reactions of beta-oxidation. This may be true for both anaerobic and aerobic conditions.

Under aerobic conditions, the metabolism of 2-aminobenzoate (1a) and 4-chlorobenzoate (24a) was shown to proceed via CoA thioesters. The role of phenylacetate-CoA ligase

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**TABLE 4. N-terminal amino acid sequences of the CoA ligases E₁, E₂, and E₃ acting on benzoate and 2-aminobenzoate**

<table>
<thead>
<tr>
<th>CoA ligase</th>
<th>N-terminal amino acid sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>E₁</td>
<td>Ala Glu Leu Ser Val Ala Asp (His) (Ser) Val (X) Pro Pro</td>
</tr>
<tr>
<td>E₂</td>
<td>Thr Ser His Val Asp Thr Phe Ala (Arg) Asp (Arg) (X) Pro Pro (Thr) Glu Gin Gin (Thr) Glu (Ser) Leu</td>
</tr>
<tr>
<td>E₃</td>
<td>Thr Ser His Val Asp Thr Phe Ala Arg Asp (X) Leu Pro Pro (X) (Glu) Gin Gin</td>
</tr>
</tbody>
</table>

* This residue appears not to be Leu.
aerobically expressed in a P. putida strain is unknown (22); it has been hypothesized that it is involved in a novel aerobic phenylacetate metabolism, although its kinetic constants are not in favor of such a function.

The three enzymes reported here have properties similar but not identical to those of the benzoate-CoA ligase and 4-hydroxybenzoate-CoA ligase from R. palustris first isolated by Gibson and coworkers; therefore, many aspects of these enzymes have been discussed previously (10). The enzymes belong to a group of carboxylate-CoASH ligases (AMP forming) (EC 6.2.1) with a relatively high specificity for aromatic substrates and little or no activity toward nonaromatic acids. With respect to high nucleotide specificity, E1, E2, and E3 are comparable to most other investigated CoA ligases, but not to phenylacetate-CoA ligase of P. putida, which reacts weakly also with ADP, CTP, or UDP (22), or acetate-CoA ligase of Bradyrhizobium japonicum, which was reported also to activate acetic acid with ADP and dATP (26).

The benzoate-CoA ligase E1 activates only a small number of structurally related aromatic acids. Significant activities were observed only with benzoate, 2-fluorobenzoate, and 4-fluorobenzoate; little activity was found with 3-fluorobenzoate and 2-amibenzoate. The 2-amibenzoate-CoA ligases E2 and E3 activated 2-amibenzoate, benzoate, 2-fluorobenzoate, and 4-fluorobenzoate; with 3-fluorobenzoate and 2-methylbenzoate, little activity was observed. Of the enzyme substrates tested, benzoate, 2-amibenzoate, 2-fluorobenzoate, 3-hydroxybenzoate, 4-hydroxybenzoate, phenylacetate, cyclohexanecarboxylate, and acetate have been reported to be growth substrates (3, 28, 34). It is likely that most if not all of these organic acids are metabolized via their CoA thioesters. This again suggests that several other synthetases exist which may be induced by their specific substrates.

The apparent molecular mass of the benzoate-CoA ligase E1 as determined by gel filtration is 120 kDa; the enzyme is a homodimer with an estimated subunit mass of 55 kDa. This is approximately the molecular mass of the following monomeric enzymes: benzoate-CoA ligase (60 kDa) of R. palustris (10), the 2-amibenzoate-CoA ligases E2 (60 kDa) and E3 (65 kDa), and phenylacetate-CoA ligase in Pseudomonas strain KB 740” (61 kDa) (22). The homodimeric acetate-CoA ligase of B. japonicum (26) has a molecular mass of 150 kDa. The pH optima of all three CoA ligases range from pH 8.5 to 9.2, which are the same as the values reported for other investigated CoA ligases (10). This may reflect the requirement for the CoA thiolate anion (pK 8) as a nucleophile in catalysis. The enzymes were inhibited by agents that react with thiol groups. This may suggest that SH or thiolate groups are essential for the catalytic activity, e.g., in the formation of an S-acylated enzyme intermediate after the adenylic acid has been transferred to the aromatic acid.

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REFERENCES

AROMATIC ACID-COENZYME A LIGASES


