Involvement of carbon dioxide in the aerobic biodegradation of ethylene oxide, ethene, and vinyl chloride

Anthony S. Danko a,*, David L. Freedman b

a Institute for Biotechnology and Bioengineering, Centro de Engenharia Biológica, Universidade do Minho, Campus Gualtar, Braga 4710-057, Portugal
b Department of Environmental Engineering and Science, Clemson University, Clemson, SC 29634, USA

Received 8 September 2007; received in revised form 4 January 2008; accepted 11 January 2008

Abstract

The involvement of a carboxylase in metabolism of C-2 alkenes by Ochrobactrum sp. strain TD and Pseudomonas putida strain AJ was examined. With resting cells of strain TD grown on vinyl chloride, ethene, and ethylene oxide, the maximum specific rate of ethylene oxide consumption decreased significantly in the absence of external CO2 in comparison to cells provided with room air or added CO2. The amount of 14CO2 incorporated into biomass by resting cells of strain TD grown on ethylene oxide increased more than 13-fold when the assay substrate was ethylene oxide versus acetate. These results indicate that strain TD uses a carboxylase. Similar experiments were performed with strain AJ with the results suggesting that a carboxylase is not involved. In this regard, strain AJ is more similar to various Mycobacterium isolates that also do not appear to use a carboxylase during metabolism of vinyl chloride and ethene.

#2008 Elsevier Ltd. All rights reserved.

Keywords: Vinyl chloride; Ethene; Ethylene oxide; Carboxylase; Carbon dioxide; Biodegradation

1. Introduction

The involvement of coenzyme M (CoM) during aerobic metabolism of propylene has been demonstrated in Xanthobacter strain Py2 and Rhodococcus rhodochrous strain B276. The pathway involves epoxypolyene:CoM transferase, dehydrogenase, and oxidoreductase and carboxylase reactions, yielding acetoacetate as a product [1–4]. The monooxygenase and epoxide carboxylase genes for catabolism of propylene are located on a large linear plasmid (320 kb), although these genes are not clustered together [5]. Catabolism of acetone, an isomer of propylene oxide (PrO), also involves carboxylase activity under aerobic and anaerobic conditions [6].

Various bacteria have been isolated that use vinyl chloride (VC) and ethene as sole sources of carbon and energy under aerobic conditions, including several strains of Pseudomonas [7–9] and Mycobacterium [10], one strain of Nocardioiodes [10], one strain of Ralstonia [28], and one strain of Ochrobactrum [9]. As with propylene, CoM is involved in the catabolic pathway for ethene and VC in several strains of Mycobacterium [11,12] and in one strain each of Pseudomonas, Ochrobactrum [13], and Nocardioiodes [14]. Coleman and Spain [12] demonstrated that the product of epoxypolyene:CoM transferase from ethylene oxide (EtO) is 2-hydroxyethyl-CoM, although they did not evaluate the pathway beyond this intermediate. 2-Chloro-2-hydroxyethyl-CoM was predicted to be the product from VC-epoxide. No evidence was found for the presence of epoxide carboxylase genes near the epoxypolyene:CoM transferase gene in Mycobacterium strain JS60. Conversion of hydroxyethyl CoM to acetyl CoA was predicted to proceed without incorporation of CO2 [12], as first suggested based on biochemical studies with Mycobacterium strain E20 [15].

Sequencing results for linear plasmid DNA from Nocardioiodes strain JS614 revealed several genes involved in alkene metabolism including an alkene monooxygenase, epoxypolyene:CoM transferase, CoA transferase, acyl-CoA synthetase, dehydrogenase, reductase, and possible CoM biosynthesis genes [14]. New evidence suggests that a carboxylase-like protein was expressed in response to VC, ethene, and ethylene oxide in Nocardioiodes strain JS614 [16]. However, no direct biochemical tests were conducted for the presence of a carboxylase in strain JS614.

* Corresponding author. Tel.: +351 253604400; fax: +351 253678986. E-mail address: asdanko@deb.uminho.pt (A.S. Danko).

1359-5113/$ – see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.procbio.2008.01.008
Because CoM is involved in aerobic catabolism of VC, ethene, and EtO, we hypothesized that carboxylase activity may be involved in the pathway for some of the microbes that utilize these substrates, analogous to what occurs with propylene in strains B276 and Py2. We evaluated Ochrobactrum sp. strain TD and P. putida strain AJ, which we previously isolated and characterized with respect to growth on VC, ethene and EtO [9] and the involvement of CoM in the degradation pathway [13].

2. Materials and methods

2.1. Chemicals and media

VC gas (99.5%) was purchased from Fluka (Milwaukee, WI); ethene (99.9%) from Matheson (Montgomeryville, PA); and EtO (99.5%) and PrO (99.9%) from Sigma–Aldrich (Milwaukee, WI). NaH14CO3 was purchased from ICN Radiochemicals (50 mCi/mmol) (Irving, CA). All other chemicals used were of reagent grade. The mineral salts medium used to grow strains AJ and TD is described in Hartmans et al. [17], except that the amount of (NH4)2SO4 was reduced to 0.67 g L−1. No vitamins or other complex growth factors were added to the mineral salts medium. The nitrate mineral salts medium used for growth of R. rhodochrous strain B276 is described in Whittembury et al. [18].

2.2. Analytical methods

The total amount of VC, ethene, EtO, propylene, and PrO in culture and serum bottles was determined by gas chromatographic analysis of headspace samples, as previously described [7]. Protein was measured in the supernatants using the bicinchoninic acid–copper assay with bovine serum albumin as a standard [19]. Protein concentrations were determined by lysing cells at 65°C using an Aminex HPX-87H column (300 mm × 8 mm; Bio-Rad, Herculues, CA), a 5 mM H2SO4 mobile phase, and a UV–Vis detector (210 nm; Waters 490E).

Glucose was measured by chemical oxygen demand using the Hach method (Hach Company, Loveland, CO; kit range = 5–150 mg L−1). Acetic acid was measured by high performance liquid chromatography (Waters 717, 600E) using an Aminex HPX-87H column (300 mm × 7.8 mm) and a Micro-Guard cartridge (30 mm × 4.6 mm; BIO-RAD, Hercules, CA), a 5 mM H2SO4 mobile phase, and a UV–Vis detector (210 nm; Waters 490E).

2.3. Cultures and growth conditions

Cultures were grown at 30°C in 0.725 L, 2.3 L, or 2.5 L glass bottles. The bottles that were used to grow cultures on EtO, ethene, or VC contained mineral salts medium that filled 28% of the total volume, while those used to grow cultures on propylene contained mineral salts medium that filled 20% of the total volume. The initial amount of ethene and propylene provided was 10% of the headspace volume. The initial amount of EtO and VC provided was 2%; higher percentages of VC were inhibitory. Repeated additions of EtO and VC were made as they were consumed, to provide enough substrate to reach the exponential growth phase; the amount of VC and EtO remaining was never allowed to decrease below 0.5% in the headspace. An adequate amount of oxygen was present initially to reach the exponential growth phase so that it did not have to be added during incubation. Cumulative substrate consumption was monitored as a function of time and used to calculate protein accumulation based on the observed protein yield [10]. From these curves it was apparent how much substrate had to be consumed to reach the exponential growth phase. Cells were harvested (see below) during the exponential growth phase for the maximum utilization rate and CO2 incorporation experiments.

2.4. Effect of CO2 on maximum substrate utilization rates

Strains AJ, TD, and B276 were grown from an initial OD600 of 0.04–0.085 to exponential phase (estimated OD600 of 0.2–0.8, based on correlation of growth of strain TD. Removal of CO2 gas (99.5%) was purchased from Fluka (Milwaukee, WI); ethene (99.9%) from Matheson (Montgomeryville, PA); and EtO (99.5%) and PrO (99.9%) from Sigma–Aldrich (Milwaukee, WI). NaH14CO3 was purchased from ICN Radiochemicals (50 mCi/mmol) (Irving, CA). All other chemicals used were of reagent grade. The mineral salts medium used to grow strains AJ and TD described in Hartmans et al. [17], except that the amount of (NH4)2SO4 was reduced to 0.67 g L−1. No vitamins or other complex growth factors were added to the mineral salts medium. The nitrate mineral salts medium used for growth of R. rhodochrous strain B276 is described in Whittembury et al. [18].

2.5. Incorporation of 14CO2 into cell suspensions

Stains AJ and TD were grown on EtO and strain B276 was grown on propylene to exponential phase. The cells were then centrifuged and resuspended in serum bottles containing 10 ml KP, as described above. Resting cells are defined as cultures that have been washed and suspended into KP buffer. This was done to minimize protein synthesis. Incorporation of 14CO2 into biomass was determined as previously described [20]. NaH14CO3 was added from a stock solution to give a specific radioactivity of 59 µCi/mmol of CO2– NaHCO3. The solutions were incubated with the resting cells for 2 min followed by addition of substrate (EtO, PrO, acetate, or glucose). Assays were prepared in duplicate with one set of serum bottles containing 14CO2 while the other set did not. When substrate consumption was complete, a 0.1 ml sample of the cell solution was removed from the cultures containing NaH14CO3 and passed through two filters (Whatman GF-A fiber filter placed on top of a 2.5 cm Super filter). The filters were washed four times with 50 mM NaHCO3– K2HPO4 (pH 8.0) and placed in liquid scintillation cocktail (Scinti Safe PlusTM 50%). Samples were counted for radioactivity with a Packard 2550 TA/RB liquid scintillation analyzer.

3. Results

3.1. Effect of CO2 on maximum substrate utilization rates

The maximum specific rate of EtO consumption by resting cells of strain AJ grown on ethene was not affected by CO2 removal (Fig. 1a) or addition of CO2 (Fig. 1b), suggesting that CO2 is not a reactant in the catabolic pathway. When only room air was available, the rate of EtO removal was similar to the treatment with CO2 added (Fig. 1c). Different results were obtained with ethene-grown strain TD. Removal of CO2 significantly slowed the rate of EtO consumption (Fig. 1d) in comparison to adding CO2 (Fig. 1e) or providing only room air (Fig. 1f). These results suggest CO2 may be involved as a reactant in the catabolic pathway of strain TD.

Maximum specific EtO utilization rates for strains AJ and TD grown on VC, ethene and EtO are summarized in Table 1. The type of growth substrate did not alter the pattern of the results described above. With strain AJ, the presence or absence of CO2 did not have a significant affect on the rate of EtO utilization. With strain TD, removal of CO2 by trapping in KOH reduced the rate of EtO consumption by one order of magnitude; adding CO2 above what was present in room air did not alter the rate of EtO use.
Strain B276 was used as a positive control since previous studies showed it incorporates CO₂ during catabolism of PrO [21]. As with strain TD, removal of CO₂ significantly decreased the maximum rate of substrate utilization by strain B276, although providing an excess above what was available in room air did not stimulate the utilization rate (Table 1). A decrease in the maximum specific rate of PrO consumption during the same type of resting cell assay, when CO₂ was not available, has also been shown with propylene-grown Xanthobacter strain Py2 [20].

Air was present in the headspace of the serum bottles during the whole cell assays, so that oxygen was available to the cells. When air was replaced with nitrogen to minimize the availability of internally generated CO₂ (via mineralization), very little EtO consumption occurred with strains AJ and TD (data not shown).

3.2. Incorporation of ¹⁴CO₂ into cell suspensions

Incorporation of ¹⁴CO₂ into resting cells is summarized in Table 2. The amount of ¹⁴CO₂ incorporated by strain TD grown on EtO was more than 13 times higher when EtO was the substrate compared to acetate (from 0.18 to 2.47 mol CO₂ per mol of substrate), the catabolism of which does not require CO₂. With strain AJ, there was no significant difference between the amount of CO₂ incorporated when the substrate was EtO or acetate. Approximately twice as much CO₂ was incorporated by strain B276 when the assay substrate was PrO versus glucose.

Table 2 also shows the results in terms of the amount of ¹⁴C incorporated into biomass as a percentage of the total NaH¹⁴CO₃ added. For strains AJ and TD, the amount of ¹⁴CO₂ incorporated was approximately the same when no

<table>
<thead>
<tr>
<th>Strain</th>
<th>Substrate</th>
<th>Mol CO₂ incorporated per mol substrate consumed</th>
<th>¹⁴CO₂ incorporated into biomass (% of total ¹⁴C added)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AJ</td>
<td>EtO</td>
<td>2.47 ±0.16</td>
<td>9.9 ±0.65</td>
</tr>
<tr>
<td></td>
<td>Acetate</td>
<td>0.18 ±0.04</td>
<td>0.72 ±0.14</td>
</tr>
<tr>
<td></td>
<td>None</td>
<td>–</td>
<td>0.68 ±0.07</td>
</tr>
<tr>
<td>TD</td>
<td>EtO</td>
<td>0.35 ±0.02</td>
<td>1.4 ±0.06</td>
</tr>
<tr>
<td></td>
<td>Acetate</td>
<td>0.38 ±0.01</td>
<td>1.1 ±0.02</td>
</tr>
<tr>
<td></td>
<td>None</td>
<td>–</td>
<td>0.65 ±0.12</td>
</tr>
<tr>
<td>B276</td>
<td>PrO</td>
<td>0.99 ±0.06</td>
<td>4.0 ±0.20</td>
</tr>
<tr>
<td></td>
<td>Glucose</td>
<td>0.49 ±0.05</td>
<td>1.5 ±0.20</td>
</tr>
<tr>
<td></td>
<td>None</td>
<td>–</td>
<td>1.4 ±0.10</td>
</tr>
</tbody>
</table>

* Average of duplicates; data ranges are in parentheses.
substrate was present and when acetate was added. Similar results were obtained for strain B276 when no substrate was added and when glucose was added. The amount of $^{14}$CO$_2$ incorporated with substrates whose catabolism does not involve carboxylase activity (in this case, acetate and glucose) was not significantly different from the background amount of $^{14}$CO$_2$ incorporation when no substrate was present. The significantly higher percentage of $^{14}$C activity incorporated into biomass during catabolism of EtO by strain TD and PrO by strain B276 is also apparent in Table 2.

4. Discussion

Strains TD and B276 are similar with respect to their use of CoM and a carboxylase during metabolism of alkenes. With strain B276, the expected stoichiometry is one mole of CO$_2$ incorporated per mole of PrO [3]. Our results with strain B276 are in good agreement with this expectation (Table 2), although the net amount of CO$_2$ incorporation is lower when subtracting out the background uptake based on glucose as a substrate. The stoichiometry of CO$_2$ incorporation by strain TD was approximately two moles of CO$_2$ per mole of EtO consumed, after subtracting out the amount of $^{14}$CO$_2$ incorporated when acetate was the substrate (Table 2). Based on the proposed pathway for ethene catabolism by Coleman and Spain [12], a possible pathway for EtO metabolism by strain TD would proceed by conversion to 2-hydroxyethyl CoM and oxidation to 2-ketoethyl-CoM followed by addition of one mole of CO$_2$ to each carbon as CoM is released. The presumptive C-4 product would be oxaloacetate, with no net gain or loss of reducing power. A similar pathway may occur when VC is converted to 2-chloro-2-hydroxyethyl CoM, although its conversion to 2-ketoethyl-CoM involves an elimination reaction rather than an oxidation, so a net input of reducing power would be required to form oxaloacetate from VC.

Considering that strain TD appears to incorporate CO$_2$ during EtO catabolism, it is not yet known why oxygen was required for EtO utilization during the whole cell assay even when an exogenous source of CO$_2$ was provided. One possibility is the need for ATP to drive EtO uptake or CO$_2$ incorporation. The overall conversion of 1 mole of EtO plus 2 moles of CO$_2$ to 1 mole of oxaloacetate is exergonic at the assay pH of 7.2 (based on free energies of formation from Lide [22]; Madigan et al. [23]; Reid et al. [24]; Voet and Voet [25] and a Henry’s law constant for ethylene oxide from Reid et al. [24]). Nevertheless, one of the steps in the pathway may be endergonic and require an input of ATP. Carboxylase activity in acetone-grown strain Py2 and strain B276 is dependent on the availability of ATP and GTP, respectively [26,27]. Resting cell assays of strain Py2 and B276 were not tested in the absence of oxygen to determine if the reaction with acetone could proceed without oxygen. It is also possible that the carboxylase-like enzyme in strain TD functions differently than the one found in strain B276.

The substrate utilization assay and CO$_2$ incorporation results indicate that a carboxylase is not involved in the pathway for EtO catabolism used by strain AJ. When growing on VC and ethene, strain AJ appears to use a non-carboxylase pathway that may be similar to the one proposed for various *Mycobacterium* strains [12] and *Nocardioides* strain JS614 [14] via the formation of carboxymethyl-CoM. The gene organization in *Mycobacterium* strain JS60 based on sequencing results suggest the involvement of a CoA transferase and acyl-CoA synthetase [12]. These enzymes are located directly upstream from the alkene monoxygenase and CoM transferase genes. Coleman and Spain [12] suggested coenzyme A transferase and synthetase transfer coenzyme A onto 2-ketoethyl-CoM while removing CoM. Mattes et al. [14] further elucidated this pathway in VC and ethene-grown *Nocardioides* strain JS614.

In summary, the results of this study provide evidence that strain TD uses a carboxylase during catabolism of EtO (and by extension ethene and VC), while strain AJ does not. Additional studies are needed to further characterize the pathways. From an application standpoint, identification of the enzyme(s) that are unique to VC metabolism is a high priority (if indeed such enzymes exist), in order to provide a basis for distinguishing between the potential for VC and ethene biodegradation in aerobic environments.

Acknowledgements

Dr. Tom Smith (Department of Biomedical and Molecular Sciences, Sheffield Hallam University) generously provided *Rhodococcus rhodochrous* strain B276.

References


