Biosynthesis of amino acids by Oxalobacter formigenes: analysis using 13C-NMR

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Abstract
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Keywords
oxalate, carbon flow, carbon assimilation

Disciplines
Veterinary Medicine | Veterinary Microbiology and Immunobiology

Comments
This article is from Canadian Journal of Microbiology 42 (1996): 1219, doi:10.1139/m96-157.

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Biosynthesis of amino acids by Oxalobacter formigenes: analysis using $^{13}$C-NMR

Nancy A. Cornick, Bin Yan, Shelton Bank, and Milton J. Allison

Abstract: The gram-negative anaerobe Oxalobacter formigenes, grows on oxalate as the principal carbon and energy source, but a small amount of acetate is also required for growth. Experiments were conducted to determine the distribution and the position of label in cellular amino acids from cells grown on $^{13}$C-oxalate, $^{13}$C-acetate (1-$^{13}$C, 2-$^{13}$C, and U-$^{13}$C), and $^{13}$CO$_3$. The labeling pattern (determined with NMR spectroscopy) of amino acids was consistent with their formation through common biosynthetic pathways. The majority of the carbons in the amino acids that are usually derived from pyruvate, oxaloacetate, $\alpha$-ketoglutarate, 3-phosphoglycerate, and carbon in the aromatic amino acids were labeled by oxalate. Carbon from $^{13}$CO$_3$ was assimilated primarily into amino acids expected to be derived from oxaloacetate and $\alpha$-ketoglutarate. Approximately 60% of the acetate that was assimilated into amino acids was incorporated as a C$_2$ unit into proline, arginine, glutamate, and leucine. The pattern of labeling from acetate in glutamate, arginine, and proline was consistent with acetate incorporation via citrate (si)-synthase and subsequent formation of $\alpha$-ketoglutarate via the first third of the tricarboxylic acid pathway. Acetate was also assimilated into amino acids derived from pyruvate and oxaloacetate, but results indicated that this incorporation was as single carbon atoms. Based on these findings, cell-free extracts were assayed for several key biosynthetic enzymes. Enzymatic activities found included glutamate dehydrogenase, phosphoenolpyruvate carboxylase, and pyruvate carboxylase. These findings are consistent with proposed biosynthetic mechanisms.

Key words: oxalate, carbon flow, carbon assimilation.

Résumé : L’Oxalobacter formigenes, une bactérie anaérobie gram-négative, se développe en présence d’oxalate comme seule source de carbone et d’énergie, mais elle a aussi besoin d’une faible quantité d’acétylate. Des mesures ont été faites pour déterminer la distribution et la position du marqueur dans les acides aminés retrouvés dans les cellules cultivées en présence de $^{13}$C-oxalate, $^{13}$C-acétate (1-$^{13}$C, 2-$^{13}$C, et U-$^{13}$C) et de $^{13}$CO$_3$. Le profil de marquage des acides aminés (déterminé par spectroscopie NMR) était cohérent avec leur formation selon des sentiers communs de biosynthèse. La majorité des carbones des acides aminés qui sont habituellement dérivés du pyruvate, de l’oxaloacétate, de l’$\alpha$-cétoglutarate, du 3-phosphoglycérate et du carbone des acides aminés aromatiques se retrouvaient marqués par l’oxalate. Le carbone provenant de $^{13}$CO$_3$ était d’abord assimilé dans les acides aminés qui sont prévus à partir de l’oxaloacétate et de l’$\alpha$-cétoglutarate. Environ 60% de l’acétate assimilé dans les acides aminés était sous forme d’unité C$_2$ dans la proline, l’arginine, le glutamate et la leucine. En partant de l’acétate, le profil de marquage dans le glutamate, l’arginine et la proline était conforme avec l’incorporation de l’acétate via la citrate (si)-synthase et la formation subséquente de l’$\alpha$-cétoglutarate via le premier tiers du sentier de l’acide tricarboxylique. L’acétate était aussi assimilé dans les acides aminés dérivés du pyruvate et de l’oxaloacétate, mais les résultats indiquent que ce sont des atomes de carbone simple qui sont assimilés. Suite à ces observations, quelques enzymes-clés de la biosynthèse ont été recherchées dans des extraits acellulaires. Les activités enzymatiques observées ont été la glutamate déshydrogénase, la phosphoenolpyruvate carboxylase et la pyruvate carboxylase. Nos résultats concordent avec les mécanismes proposés pour la biosynthèse.

Mots clés : oxalate, flux du carbone, assimilation du carbone.

[Traduit par la rédaction]
Introduction

*Oxalobacter formigenes* is an anaerobic gram-negative rod that has been isolated from the gastrointestinal tract of many warm-blooded animals (Allison et al. 1985; Daniel et al. 1987), including humans (Allison et al. 1986), and from anoxic sediments (Smith et al. 1985). *Oxalobacter formigenes* has an absolute dependence on oxalate as a substrate for growth. Approximately 99% of the oxalate metabolized is decarboxylated to formate and CO$_2$ in a nearly 1:1 ratio (Allison et al. 1985). The remaining oxalate (1%) is utilized for cell biosynthesis. Cell-free extracts of *O. formigenes* contain activity for the key enzymes of the glycerate pathway, wherein two molecules of oxalate are reduced to one molecule each of 3-phosphoglycerate (3P-glycerate) and CO$_2$ (Cornick and Allison 1996a). The formation of 3P-glycerate appears to be crucial for oxalate carbon to enter central metabolic pathways.

Although acetate does not support the growth of *O. formigenes*, a small amount of acetate (0.5 mM) is required (Allison et al. 1985). A number of anaerobic bacteria assimilate acetate and CO$_2$ into cell protein using the reductive C$_4$ dicarboxylic acid pathway (Buchanan 1972) or a horseshoe-type of tricarboxylic acid (TCA) pathway (Hoare and Gibson 1963). In both of these pathways acetate is assimilated as a C$_2$ unit. When *O. formigenes* was grown in [1-$_{13}$C]- or [2-$_{13}$C]-acetate, lysine from cell protein was labeled by the methyl but not by the carboxyl carbon of acetate (Cornick and Allison 1996b). The relative specific activity of alanine compared with that of glutamate was similar when cells were grown with [2-$_{14}$C]acetate but was much less (33%) when cells were grown with [1-$_{14}$C]acetate. These results suggest that acetate was not always assimilated into amino acids as a C$_2$ unit. To gain additional insight into the metabolism of *O. formigenes*, we grew it in [1$_{13}$C]oxalate, [1-$_{13}$C]acetate, [2-$_{13}$C]acetate, [U-$_{13}$C]acetate, and 13CO$_2$ and determined the extent and position of the 13C-label in amino acids. We also assayed cell-free extracts for the following enzymatic activities: pyruvate carboxylase, phosphoenolpyruvate (PEP) carboxylase, glutamate dehydrogenase, NADH-linked malate dehydrogenase (Sigma Chemical Co., St. Louis, Mo.) was used (rather than NADPH linked). Protein was measured using a modified Lowry assay (Peterson 1977) with bovine serum albumin as the standard.

Materials and methods

Bacteria

*Oxalobacter formigenes* (ATCC 35274) was grown in 1-L batches (0.5% inoculum) under a N$_2$ gas phase in medium E, a defined synthetic medium (Cornick and Allison 1996b) containing 100 mM oxalate and 1 mM [1-$_{13}$C]acetate, 1 mM [2-$_{13}$C]acetate, 1 mM [U-$_{13}$C]acetate, or 24 mM Na$_3$CO$_3$. For cultures exposed to 13CO$_2$, the pH of the medium was adjusted to 5.8 using filter-sterilized 1 N HCl. Cells labeled with [1$_{13}$C]oxalate (26% enriched, 70 mM) were grown under a CO$_2$ gas phase and in a carbonate buffer system (medium F; Cornick and Allison 1996b) to dilute and minimize the contribution of 13CO$_2$, arising from the decarboxylation of [1$_{13}$C]oxalate, to cell biomass. Sterile sodium acetate was added to a final concentration of 1 mM to flasks containing labeled oxalate or CO$_3$.

Fractionation of cells

Cells were fractionated using the method of Roberts et al. (1963). Cell protein was hydrolyzed in a sealed glass tube under N$_2$ in 6 N HCl for 20 h at 105°C. The HCl was removed under vacuum. The protein hydrolysate was dissolved in D$_2$O.

NMR spectra

The spectra of all of the protein hydrolysates, except the hydrolysate from cells grown in [1$_{13}$C]oxalate, were acquired on a Varian XL-300 multinuclear spectrometer at 75.43 MHz with a 5-mm probe, using broad-band proton decoupling, a 20-KHz spectral width, and 20 032 data points. The pulse width was set at 10 µs (CO$_3$ sample, 6 µs), corresponding to a 45° flip angle. The recycle time was 2.5 s, including a 2-s delay time (CO$_3$ sample, 1 s) and a 0.5-s acquisition time. A signal/noise ratio of high quality was obtained by using 6000 – 33 000 transients.

The spectrum from the protein hydrolysate of cells grown on [1$_{13}$C]oxalate was acquired on a Varian 500 MHz UNITY spectrometer at 125.7 MHz with a 5-mm probe. The delay time was 1 s and the acquisition time was 0.5 s. The pulse width was 8 µs and 52 656 transients were collected.

Chemical shifts were referenced to tetramethylsilane using dioxane as an external standard. The identity of the signals was based on previously published values (Miller et al. 1995). When there was a question regarding the identity of the signals, unlabeled amino acid was added to the sample to enhance the signal. Owing to the nuclear Overhauser effect (NOE) and differences in relaxation times for different carbons, an intensity coefficient ratio of approximately 3:1 of the carboxyl peaks to other protonated peaks was applied to each sample to determine the semiquantitative enrichment distribution on each labeled position. The 3:1 ratio was determined by using standard amino acids under the same conditions used for the enriched samples.

Enzyme assays

Cell-free extracts were prepared using a French press as previously described (Cornick and Allison 1996a). Glutamate dehydrogenase (EC 1.4.1.4) was assayed spectrophotometrically using the method of Joyner and Baldwin (1966). Pyruvate carboxylase (EC 6.4.1.1) and PEP carboxylase (EC 4.1.1.3) were assayed by coupling the formation of oxaloacetate to the formation of malate by malate dehydrogenase (Scrutton 1971). β-Hydroxyaspartate dehydratase (EC 4.2.1.38) was also assayed by linking the formation of oxaloacetate to malate (Gibbs and Morris 1970). Since cell-free lysates of *O. formigenes* contain a negligible amount of NADH oxidase, NADH-linked malate dehydrogenase (Sigma Chemical Co., St. Louis, Mo.) was used (rather than NADPH linked). Protein was measured using a modified Lowry assay (Peterson 1977) with bovine serum albumin as the standard.

13C-labeled compounds

[1-$_{13}$C]Acetic acid (99% enriched), Na$_3$CO$_3$ (99% enriched), and sodium [U-$_{13}$C]acetate (99% enriched) were purchased from Cambridge Isotope Laboratories (Andover, Mass.). Sodium [2-$_{13}$C]acetate (92% enriched) and 13C$_2$ oxalic acid (90 and 99% enriched) were purchased from MSD Isotopes (Montréal, P.Q.). 13C$_2$ oxalic acid (99% enriched) was also purchased from IsoTech (Miamisburg, Ohio).

Results

The major labeling patterns of the acid-stable amino acids derived from [1$_{13}$C]oxalate, [1$_{13}$C]acetate, and 13CO$_2$ are shown in Fig. 1. The spectra from the oxalate-enriched sample contained approximately 20 signals in the 170- to 174-ppm range, which precluded the identification of individual carboxyl carbons. Therefore, the C1 position of each amino acid (except leucine) was presumed to be provided by oxalate.

Aspartate

Aspartate was labeled by oxalate in positions C1, C2, and C3. The C4 position was labeled by oxalate and CO$_3$. This labeling
is consistent with the carboxylation of pyruvate or PEP (which was derived from oxalate via the glycerate pathway) to form oxaloacetate. The incorporation of $^{13}$CO$_3$ arising from the decarboxylation of $[^{13}$C]oxalate would explain the labeling of the C4 position by oxalate.

**Lysine, threonine, and methionine**
The labeling of these amino acids was consistent with their derivation from aspartate. The labeling of lysine with all positions labeled from oxalate is as expected if biosynthesis proceeded via diaminopimelic acid, which is the common pathway used by bacteria. Lysine synthesized via the aminoadipic acid pathway should be labeled by the carboxyl carbon of acetate in positions C1 and C6 and by the methyl carbon of acetate in positions C2 and C5.

**Isoleucine**
The C1 position of isoleucine was labeled by both oxalate and CO$_3$. The remaining carbon skeleton was derived from oxalate. Isoleucine synthesized from threonine by the common biosynthetic pathway would result in a labeling pattern consistent with our results. The labeling of the C1 position of isoleucine by CO$_3$ indicates that *O. formigenes* may synthesize isoleucine by a second pathway or that exchange reactions occur between CO$_3$ and the carboxyl carbon of ketoisoleucine.

**Glutamate, arginine, and proline**
These amino acids were labeled by oxalate in the C1, C2, and C3 positions. The C4 and C5 positions were labeled by the methyl and carboxyl carbons of acetate, respectively. The C1 position was also labeled by CO$_3$. α-Ketoglutarate synthesized from oxaloacetate plus acetate via citrate (si)-synthase would result in glutamate having a labeling pattern matching our results. The labeling of proline and arginine indicates that they were derived from glutamate.

**Alanine, valine, and leucine**
All of the carbons from alanine and valine were labeled by oxalate. This is consistent with the usual biosynthetic pathways whereby these amino acids would be derived from pyruvate that had arisen from oxalate via the glycerate pathway. Leucine was labeled by acetate at the C1 and C2 positions, while carbons 3–6 were provided by oxalate. Leucine synthesized via the isopropylmalate pathway would result in this labeling pattern.

**Glycine and serine**
The labeling of serine was consistent with its formation from 3P-glycerate, which was derived from oxalate. The labeling of glycine is as expected if it was formed from serine.

**Phenylalanine and tyrosine**
All of the carbons of phenylalanine and tyrosine were supplied by oxalate. This labeling is consistent with known pathways of biosynthesis via shikimate and chorismate with erythrose-4-P and PEP being derived from oxalate. We did not detect $^{13}$C in histidine, which was probably due to the small amount of histidine in the protein hydrolysate.
Fig. 2. NMR spectra of amino acids from cells grown in (A) \([1-^{13}C]\)acetate, (B) \([2-^{13}C]\)acetate, and (C) \([U-^{13}C]\)acetate. 1, C5 of glutamate; 2, C1 of leucine; 3, C5 of proline; 4, C5 of arginine; 5, C2 of leucine; 6, C4 of glutamate; 7, C4 of proline; 8, C4 of arginine.
methionine (position C2), aspartate (position C3), tyrosine (position C3), and phenylalanine (position C3). The distribution of the methyl carbon of acetate ranged from 0.1% for tyrosine (position C3) to 5.3% for alanine (position C3). The carboxyl carbon of acetate was incorporated into the C1 position of isoleucine, aspartate, valine, and alanine. The distribution of the carboxyl carbon of acetate in these amino acids was 1.1, 1.5, 0.8, and 0.9%, respectively. By comparison, the distribution of the eight carbons labeled by intact acetate ranged from 12.2% for the C4 carbon of arginine to 25.2% for the C4 carbon of glutamate.

Enzyme activity
Cell-free extracts of *O. formigenes* contained both NADPH and NADH-dependent glutamate dehydrogenase, pyruvate carboxylase, and PEP carboxylase activities (Table 1). Even with these crude enzyme preparations, PEP carboxylase activity was enhanced (25%) by the addition of acetyl-CoA, but activity was not dependent on it. Pyruvate carboxylase activity was not enhanced by the addition of acetyl-CoA. β-Hydroxyaspartate dehydratase activity was not detected.

**Discussion**

The position of labeling in amino acids synthesized in the presence of 13C-labeled substrates fits well with previous enzymatic data (Cornick and Allison 1996a, 1996b), which indicated that (i) the glycerate pathway operates to synthesize 3P-glycerate and thus to incorporate carbon from oxalate into a C3 unit for utilization in biosynthetic pathways; (ii) acetate is incorporated into amino acids via citrate synthase and the first step of the TCA pathway; and (iii) the reductive C4 dicarboxylic acid pathway does not function in *O. formigenes*. The majority of the carbon (54%) that *O. formigenes* assimilates is derived from oxalate, while only 7% is derived from acetate (Cornick and Allison 1996b). The labeling of the majority of the carbons in all of the amino acids by oxalate supports the concept that oxalate is the major source of cellular carbon for this organism.

The main role of PEP carboxylase and pyruvate carboxylase in various heterotrophic bacteria is anaplerotic. The formation of C4 units allows for the continuation of the Krebs cycle when intermediates are removed for biosynthetic reactions (Utter and Kolenbrander 1972). In *O. formigenes*, the need is not for replacement of intermediates but rather for synthesis of C4 and higher compounds from oxalate, acetate, and carbonate. The heavy labeling of glutamate by acetate and the absence of a C2 unit from acetate in aspartate support previous enzymatic data (Cornick and Allison 1996b) which indicate that *O. formigenes* did not utilize a complete TCA cycle. The major route for the formation of C4 compounds is probably through PEP carboxylase and pyruvate carboxylase. PEP carboxylase is also primarily a biosynthetic enzyme in *Methanobacterium thermautotrophicum* (Kenaley and Zeikus 1982) and *Thiobacillus thiopсиданум* (Howden et al. 1972).

An alternate pathway to form C4 compounds has been noted with *Micrococcus denitrificans* (Gibbs and Morris 1964, 1970). When this organism is grown on glycolate, the enzymes of the β-hydroxyaspartate pathway catalyze the condensation of glyoxylate and glycine to form β-hydroxyaspartate. The subsequent deamination of β-hydroxyaspartate then yields oxaloacetate. In *O. formigenes*, glyoxylate is an intermediate in the formation of 3P-glycerate pathway and aspartate from this organism was labeled in all four carbons by oxalate, so the β-hydroxyaspartate pathway was a potential route for the formation of oxaloacetate. Our failure to detect β-hydroxyaspartate dehydratase activity in cell-free extracts suggests this pathway does not function in *O. formigenes*.

The labeling of glutamate in the C1 position by CO₂ and the C4 and C5 positions by acetate indicates that *O. formigenes* produces a citrate (si)-synthase (Gottschalk and Barker 1966; Tomlinson 1954). A citrate (re)-synthase would have resulted in the C1 and C2 positions of glutamate being labeled by the carboxyl and methyl carbons of acetate and the C5 position of glutamate being labeled by CO₂. Acetate was incorporated as a C2 unit into glutamate, arginine, proline, and leucine. Although these four amino acids account for 60% of the acetate assimilated into protein, the other 40% was assimilated as single carbon atoms. Our data from 13C-labeled substrates agree with previous results using 14C-labeled substrates in which lysine was labeled by the methyl carbon of acetate but not by the carboxyl carbon (Cornick and Allison 1996b). The uneven incorporation of [1-14C]acetate and [2-14C]acetate into alanine can also be explained by the 13C-labeled substrates which indicate that carbons from acetate were incorporated into this amino acid as single carbons.

All of the positions that were singly labeled by either the carboxyl or methyl carbons of acetate were also labeled by oxalate. This suggests some scrambling of label between intermediates that oxalate and acetate have in common. Preliminary data from cells grown in [14C]oxalate or [14C]acetate indicated that unidentified precursor metabolites are labeled by both oxalate and acetate but not by CO₂ (Cornick 1995). Although the metabolism of other oxalate-degrading bacteria has not been examined using 13C-NMR, scrambling of carbons has been described in several other genera of bacteria. Exchange reactions between carbonate and carboxyl carbons of α-keto acids are well known (Thauer et al. 1975). *Methanococcus jannaschii* scrambles 13C-label among the three carbons of pyruvate (Sprott et al. 1993). When the bacteria are grown in [1-13C]pyruvate, some 13C label is recovered in the C2 and C3 positions of pyruvate.

*Oxalobacter formigenes* is like many species of predominant rumen bacteria (Bryant and Robinson 1962) in that it requires acetate for growth. This suggests that in microbial habitats such as the rumen or colon, where acetate is an end product and is

### Table 1. Enzyme activities in cell-free extracts of *O. formigenes*.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Assay method</th>
<th>Specific activity&lt;br&gt;a</th>
<th>Mean of three determinations from two cell lysates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glutamate dehydrogenase</td>
<td>NADPH oxidation</td>
<td>286</td>
<td></td>
</tr>
<tr>
<td>Pyruvate carboxylase</td>
<td>NADH oxidation</td>
<td>37</td>
<td></td>
</tr>
<tr>
<td>PEP carboxylase</td>
<td>NADH oxidation</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>β-Hydroxyaspartate dehydratase</td>
<td>NAD reduction</td>
<td>32</td>
<td></td>
</tr>
</tbody>
</table>

a) nmol/(min. mg protein), mean of three determinations from two cell lysates.

**Footnotes:**

1. NA, no activity detected (limit of detection).
present at relatively high concentrations, the capacity to synthesize acetate de novo has little survival value. We reported that carbon from \(^{14}\text{C}\)-labeled acetate was incorporated mainly into the lipid and protein fractions of \textit{O. formigenes} cells (Cornick and Allison 1996b). The present results indicate that the role of intact acetate in amino acid biosynthesis is mainly related to its use in synthesis of carbon skeletons of amino acids in the glutamate family and in synthesis of leucine via the isopropylmalate pathway.

Overall, our data indicate that \textit{O. formigenes} synthesized amino acids using conventional biosynthetic pathways. Labeling was consistent with carbon from oxalate being assimilated into 3P-glycerate via the glycerate pathway and then into amino acids. Carbon dioxide was assimilated into amino acids by the carboxylation of pyruvate and PEP. The majority of carbon from acetate was incorporated into amino acids by the carboxylation of pyruvate and PEP. A significant portion of carbon from acetate was also incorporated as single carbons into amino acids usually derived from oxaloacetate and pyruvate.

\section*{Acknowledgement}

We appreciate the technical assistance of Herbert Cook.

\section*{References}


