Inhibition of acetate and propionate assimilation by itaconate via propionyl-CoA carboxylase in isocitrate lyase-negative purple bacterium \textit{Rhodospirillum rubrum}

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Abstract

Iaconate is known as a potent inhibitor of isocitrate lyase. Unexpectedly, itaconate was a strong inhibitor of acetate and propionate assimilation in isocitrate lyase-negative purple non-sulfur bacterium \textit{Rhodospirillum rubrum}. It was shown that in cell extracts of \textit{R. rubrum} itaconate inhibited propionyl-CoA carboxylase (PCC) activity. The participation of PCC in propionate assimilation in \textit{R. rubrum} is well-documented, but the inhibition of acetate assimilation suggests that PCC is also involved in acetate metabolism. PCC is one of the enzymes of the citramalate cycle, the anaplerotic pathway proposed for \textit{R. rubrum} as a substitute for the glyoxylate cycle. These results provide further support for the hypothesis of the occurrence of the citramalate cycle in \textit{R. rubrum}. PCC from other isocitrate lyase-negative phototrophs, \textit{Rhodobacter sphaeroides} and \textit{Phaeospirillum fulvum}, was not inhibited by itaconate.

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Keywords: Citramalate cycle; Iaconate; Propionyl-CoA carboxylase; \textit{Rhodospirillum rubrum}

1. Introduction

The glyoxylate cycle is a sequence of anaplerotic reactions catalyzed by the following key enzymes: isocitrate lyase (ICL), cleaving isocitrate to glyoxylate and succinate, and malate synthase condensing glyoxylate with acetyl-CoA to malate. The primary function of the cycle is to allow assimilation of acetate molecules into cellular building blocks, when acetate is supplied as a sole carbon substrate for growth [1,2]. ICL has also been described in a number of methylotrophic bacteria (so-called ICL$^{+}$ serine-pathway methylotrophs), where it participates in the conversion of acetyl-CoA to glyoxylate in the course of assimilation of C$_{1}$ compounds [3,4]. The glyoxylate cycle can be strongly inhibited by itaconate. This compound acts as a potent competitive inhibitor of ICL with respect to succinate ($K_i = 0.9$ $\mu$M at a pH of 6.8 and 30$^\circ$C for the enzyme from \textit{Pseudomonas indigofera}) [5]. Iaconate suppresses the growth of bacterial cultures, when functioning of ICL is obligatory for growth (e.g., on acetate), but does not inhibit growth of the same bacteria when growth does not require ICL (e.g., on glucose) [6]. The latter is thought to provide a convenient way to detect the occurrence of ICL in vivo. In this capacity itaconate has been used for the study of anaplerotic sequences in a number of bacteria [7-10], some fungi [11], plants [12], and nematodes [13]. In all these cases the effect of itaconate correlated with participation of ICL in metabolism.

There is however a large group of bacteria that apparently lacks ICL, but can grow under conditions where an anaplerotic sequence replacing the glyoxylate cycle is necessary to sustain growth (so-called ICL$^{-}$ bacteria). Examples are methylotrophic bacteria (ICL$^{-}$ serine pathway methylotrophs, e.g., \textit{Methylobacterium extorquens} AM1) [14,15], phototrophic bacteria (e.g., \textit{Rhodospirillum rubrum}, \textit{Phaeospirillum fulvum} and \textit{Rhodobacter sphaeroides}) [16,17], unicellular sulfur bacteria (\textit{Thiobacillus versutus}; ICL$^{-}$ when grown aerobically, although ICL$^{+}$ during anaerobic growth with NO$_{3}^{-}$) [18], and streptomycetes (\textit{Streptomyces collinus}; ICL$^{-}$ when grown on acetate, although ICL$^{+}$ when grown on Tween) [19]. The exact sequence of anaplerotic reactions which ICL$^{-}$ bacteria use to augment ICL deficiency is not known, despite the fact that the enig-
ma has been pronounced over 30 years ago. Several pathways for conversion of acetate to glyoxylate in ICL\(^{-}\) methylo trophs have been proposed [20-22], but none of them have been confirmed. Recently Korotkova et al. [15] have suggested the existence of a novel glyoxylate regeneration pathway in \(M.\) extorquens AM1 involving intermediates and enzymes of poly-\(\beta\)-hydroxybutyrate synthesis, valine degradation, and polyketide biosynthesis. The first step of this pathway is condensation of two acetyl-CoA molecules to form acetoacetyl-CoA, which is (in its turn) transformed to malyl-CoA via the following intermediates: (R)-\(\beta\)-hydroxybutyryl-CoA, crotonyl-CoA, butyryl-CoA, ethylmalonyl-CoA, methylsuccinyl-CoA, isobutyryl-CoA, methacrylyl-CoA, \(\beta\)-hydroxyisobutyryl-CoA, propionyl-CoA, methylmalonyl-CoA and succinyl-CoA. The cleavage of malyl-CoA leads to acetyl-CoA and glyoxylate. It was assumed that the same or a similar pathway functions in \(S.\) spp. during growth on \(C_2\) compounds [15,19]. Pathways of \(C_2\) assimilation in other ICL\(^{-}\) bacteria remain to be identified.

We have recently proposed operation of a new cyclic pathway for acetate oxidation to glyoxylate in \(R.\) rubrum (Fig. 1) [23,24]. In this pathway named citramalate cycle, acetyl-CoA is condensed with pyruvate to form citramalate (2-methylmalate), which is then converted to propionyl-CoA and glyoxylate via the following sequence of reactions: citramalate \(\rightarrow\) mesaconate \(\rightarrow\) mesaconyl-CoA \(\rightarrow\) erythro-\(\beta\)-methylmalyl-CoA \(\rightarrow\) glyoxylate + propionyl-CoA. Propionyl-CoA is carboxylated by propionyl-CoA carboxylase (PCC) to methylmalononyl-CoA, which is converted to succinyl-CoA. The latter is transformed to oxaloacetate using part of the tricarboxylic acid cycle. Oxaloacetate is decarboxylated to phosphoenolpyruvate and converted to pyruvate, the acetyl-CoA acceptor for citramalate synthesis.

It was shown earlier, that itaconate inhibits growth on acetate or on methyamine only of those methylo trophic organisms that possess the ICL\(^{+}\) serine pathway, whereas those possessing the ICL\(^{-}\) serine pathway were unaffected [7]. Our interest in itaconate inhibition of \(C_2\) metabolism arose from the observation that this compound was a strong inhibitor of acetate assimilation in \(R.\) rubrum [24]. However, the fact that \(R.\) rubrum lacks ICL renders the effect of itaconate as rather puzzling. We proposed that itaconate can inhibit one of the enzymes of the proposed citramalate cycle [24]. The aim of this study was to provide further inside on this issue.

2. Materials and methods

2.1. Strains and culture conditions

\(R.\) rubrum strain 2R, \(Rb.\) sphaeroides strain 2R and \(P.\) fulvum strain 5K were obtained from the Collection of the Department of Microbiology, Moscow State University (KM MGU 301, 284 and 325, respectively). Cells were grown phototrophically at 2000 lux and 28\(^\circ\)C using the medium of Ormerod [25] supplemented with sodium acetate or sodium propionate (1 g l\(^{-1}\)), and sodium bicarbonate (2 g l\(^{-1}\)). In some cases, sodium malate (1 g l\(^{-1}\)) was used as a sole carbon source.

2.2. Preparation of cell extracts

Cells from mid-exponential cultures were harvested, washed with 50 mM Tris/HCl buffer (pH 7.8), resuspended in the same buffer containing 5 mM dithioerythritol, frozen at \(-20^\circ\)C, and broken by passing through an X-pressure cell. Debris was spun down at 30,000 \(\times\) g for 20 min (4\(^\circ\)C) and the resulting supernatant (cell extract) was used in enzymatic assays.

2.3. PCC assay

PCC (EC 6.4.1.3) was assayed radiochemically by determining propionyl-CoA-dependent fixation of radiolabeled bicarbonate [26]. The assay mixture (0.1 ml) contained 50 mM Tris/HCl, pH 7.8, 5 mM dithioerythritol, 6 mM MgCl\(_2\), 15 mM (0.04 MBq) NaH\(^{14}\)CO\(_3\), 2 mM ATP, 20 \(\mu\)g ml\(^{-1}\) biotin, 0.1–0.5 mM propionyl-CoA and cell extract (0.5–2.0 mg protein ml\(^{-1}\)).

2.4. Whole cell studies

Cells from early exponential cultures were harvested,
washed, and resuspended (0.04–1.5 mg protein ml\(^{-1}\)) in the basal mineral medium lacking NaHCO\(_3\). Cell respiration was measured polarographically with a Clark electrode. \(^{14}\)C assimilation was assayed in the cell suspensions incubated in glass syringes placed under uniform illumination (2000 lux). After 30 min preincubation with substrates and itaconate (where applicable), the reaction was started by the addition of \([^{14}\text{C}]\)substrate (NaH\(^{14}\)CO\(_3\), \([^{2}\text{14}\text{C}])\)sodium acetate, \([^{1,4}\text{14}\text{C}])\)sodium propionate or \([^{1,4}\text{14}\text{C}])\)sodium succinate; 0.02–0.04 MBq), and stopped at fixed time intervals by quick filtration of 1 ml suspension through 0.45-\(\mu\)m nitrocellulose filters. Filters were washed with 15 ml acidified water in order to remove unincorporated \([^{14}\text{C}]\)substrate, dried and counted in a LKB RaBeta model 1127 liquid scintillation counter.

2.5. Determination of protein concentration

Protein was measured according to the Lowry method, using bovine serum albumin as standard.

3. Results and discussion

3.1. PCC in \(R.\) rubrum

\(R.\) rubrum cells grown phototrophically on acetate, malate or propionate synthesize PCC with specific activities of 7.2, 6.6 and 23.6 nmol min\(^{-1}\) (mg protein\(^{-1}\)) respectively. PCC catalyzes the conversion of propionyl-CoA to methylmalonyl-CoA: propionyl-CoA+HCO\(_3\) +ATP\(\rightarrow\) (S)-methylmalonyl-CoA+ADP+Pi. (S)-Methylmalonyl-CoA, the product of the PCC reaction, racemizes to the (R)-enantiomer, which undergoes rearrangement to succinyl-CoA (an intermediate of tricarboxylic acid cycle). PCC functions in catabolism of odd-chain fatty acids and several amino acids [27]. The enzyme was proposed to participate in autotrophic CO\(_2\) assimilation in \(Chloroflexus\) aurantiacus and some archaea of the phylum Crenarchaeota [28–31]. PCC is also employed by many, but not all, organisms for propionate assimilation [32,33]. The participation of PCC in propionate assimilation in \(R.\) rubrum is well-documented [26,34]. Also PCC is involved in the citramalate cycle, the apolarotic pathway proposed for this bacterium [23,24]. Interestingly, PCC is required for acetyl-CoA oxidation to glyoxylate in \(M.\) extorquens [15,35].
3.2. In vitro and in vivo inhibition of R. rubrum PCC by itaconate

Itaconate competitively inhibited (Fig. 2) PCC of R. rubrum with respect to propionyl-CoA ($K_i = 0.6$ mM). Moreover, addition of itaconate completely inhibited $[2-^{14}C]$propionate assimilation in the cells grown on propionate or acetate (Fig. 3). Propionate-dependent $^{14}$CO$_2$ assimilation was also repressed in the presence of itaconate (Fig. 3). By contrast, itaconate has a subtle effect on $[1,4-^{14}C]$succinate fixation by whole cells of R. rubrum (Fig. 3). Corresponding data were obtained in the study of respiration (Table 1). Addition of itaconate substantially inhibited oxygen consumption in the presence of propionate and bicarbonate (Fig. 3). Propionate-dependent $^{14}$CO$_2$ assimilation was also repressed in the presence of succinate. Since succinate is an intermediate of propionate assimilation in the PCC pathway, these data indicate that itaconate selectively inhibits propionate oxidation at the step catalyzed by PCC. However, extracellular concentration required for inhibition of propionate assimilation is much lower than that required for effective in vitro inhibition of PCC. A possible explanation is the presence of an active transport system using itaconate in R. rubrum, which leads to an accumulation of itaconate in the cells. The presented data do not allow us to exclude the possibility that three other enzymes involved in the conversion of propionyl-CoA to succinate, methylmalonyl-CoA epimerase, methylmalonyl-CoA mutase and succinate thiokinase may also be the target of itaconate, increasing its effect on propionate assimilation.

Iaconate has been shown to also be a strong inhibitor of ICL, a key enzyme in the glyoxylate cycle for acetate assimilation. In cell extracts of R. rubrum ICL activity could not be detected [16,17,23]. In addition glyoxylate is secreted during growth on acetate, which was increased after addition of fluoroacetate [23,24]. This is not expected, if acetate is assimilated via the glyoxylate cycle. In cells with an operative TCA cycle, fluoroacetate is converted to fluoroacetyl-CoA and then to fluorocitrate [36]. The latter inhibits aconitase which is one of the enzymes of glyoxylate cycle but is not involved in acetyl-CoA oxidation to glyoxylate through the citramalate cycle. Moreover, a search of the database of the almost completed genome of R. rubrum (http://spider.jgi-psf.org/JGI_microbial/html/rhodospirillum/rhodospir_homepage.html) at the DOE Joint Genome Institute (University of California, CA, USA) revealed the absence of homologs to ICL in R. rubrum.

3.3. Itaconate inhibition of acetate assimilation in R. rubrum

Iaconate inhibited acetate fixation by R. rubrum grown in the presence of acetate (Fig. 3B). It also repressed oxygen consumption in the presence of acetate and NaHCO$_3$ (Table 1). Inhibition of acetate assimilation by itaconate suggests that PCC is involved in acetate metabolism. Since PCC is one of the enzymes of the citramalate cycle, these data provide further support for the hypothesis of the occurrence of the citramalate cycle in R. rubrum. The effect of itaconate differs from the effect of another C$_5$-dicarboxylic acid, citramalate. As we previously showed, citramalate decreased the rate of $[14C]$acetate fixation by more than 70% [23,24]. However, in contrast to itaconate it did not affect respiration of the cells in the presence of acetate and bicarbonate (Table 1). These data may be due to the substitution of acetate with citramalate as an intermediate of the citramalate cycle that results in dilution of the $[14C]$acetate label. The decrease of acetate fixation by

<table>
<thead>
<tr>
<th>Additives</th>
<th>Growth substrate</th>
<th>Propionate</th>
<th>Acetate</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (endogenous respiration)</td>
<td></td>
<td>3.4</td>
<td>4.3*</td>
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<tr>
<td>Propionate</td>
<td>18.7</td>
<td>10.8*</td>
<td></td>
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<tr>
<td>Propionate+NaHCO$_3$</td>
<td>38.5</td>
<td>20.7*</td>
<td></td>
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<tr>
<td>Propionate+NaHCO$_3$+itaconate, 1 mM</td>
<td>12.6</td>
<td>4.2*</td>
<td></td>
</tr>
<tr>
<td>Succinate</td>
<td>40.4</td>
<td>34.9</td>
<td></td>
</tr>
<tr>
<td>Succinate+itaconate</td>
<td>36.8</td>
<td>40.7</td>
<td></td>
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<tr>
<td>Acetate</td>
<td>13.8</td>
<td>8.5*</td>
<td></td>
</tr>
<tr>
<td>Acetate+NaHCO$_3$</td>
<td>19.8</td>
<td>21.8*</td>
<td></td>
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<tr>
<td>Acetate+NaHCO$_3$+itaconate, 1 mM</td>
<td>6.5</td>
<td>8.1*</td>
<td></td>
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<tr>
<td>Acetate+NaHCO$_3$+L-citramalate</td>
<td>NA*</td>
<td>27.2*</td>
<td></td>
</tr>
</tbody>
</table>

The measurements were done in darkness. The concentrations of acetate, propionate, succinate, L-citramalate and NaHCO$_3$ were 5 mM.

*From [24].

NA, Not assayed.

Table 2

<table>
<thead>
<tr>
<th>Organism and growth conditions</th>
<th>Specific activity (nmol min$^{-1}$ (mg protein)$^{-1}$)</th>
<th>Relative specific activity (in %) in the presence of itaconate at (mM):</th>
<th>0</th>
<th>0.5</th>
<th>5.0</th>
<th>10.0</th>
<th>20.0</th>
</tr>
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<tbody>
<tr>
<td>R. rubrum</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Propionate</td>
<td>23.6</td>
<td></td>
<td>100</td>
<td>84.6</td>
<td>55.4</td>
<td>36.2</td>
<td>23.0</td>
</tr>
<tr>
<td>Acetate</td>
<td>7.2</td>
<td></td>
<td>100</td>
<td>84.1</td>
<td>54.7</td>
<td>32.7</td>
<td>20.6</td>
</tr>
<tr>
<td>Acetate+itaconate</td>
<td>17.3</td>
<td></td>
<td>100</td>
<td>96.1</td>
<td>56.5</td>
<td>29.6</td>
<td>14.3</td>
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<tr>
<td>P. fulvum, propionate</td>
<td>57.8</td>
<td></td>
<td>100</td>
<td>102.1</td>
<td>90.0</td>
<td>84.9</td>
<td>79.1</td>
</tr>
<tr>
<td>Rh. sphaeroides, propionate</td>
<td>15.0</td>
<td></td>
<td>100</td>
<td>86.3</td>
<td>81.5</td>
<td>81.0</td>
<td>79.1</td>
</tr>
</tbody>
</table>

3.5. Effect of itaconate on PCC from other phototrophs

PCC from other ICL\textsuperscript{−} phototrophs, \textit{R. sphaeroides} and \textit{P. fulvum}, was not inhibited by itaconate (Table 2). Consequently, itaconate has only a subtle effect on propionate fixation by cells of these bacteria (Table 3). The respiration of the cells in the presence of propionate was not affected as well (Table 3). Although the mechanism of propionate assimilation in these bacteria has not been studied throughout, the available data indicate the participation of PCC in this process ([38], Filatova, L.V., Berg, I.A. and Ivanovsky, R.N., unpublished results). Therefore, PCC appears to be a target for itaconate in \textit{R. rubrum}, but not in \textit{Rb. sphaeroides} or \textit{P. fulvum}.

In summary, the results obtained in this study support the suggestion of a participation of PCC in acetate assimilation in \textit{R. rubrum} and, therefore, corroborate the operation of the proposed citramalate cycle in this bacterium. They also demonstrate that it is necessary to be cautious in interpreting experimental data on in vivo inhibition of ICL by itaconate in other organisms. An inhibition of growth by itaconate does not necessarily mean the operation of the glyoxylate cycle in a particular organism.

Acknowledgements

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References


Table 3

<table>
<thead>
<tr>
<th>Compounds added</th>
<th>\textit{P. fulvum}</th>
<th>\textit{R. sphaeroides}</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Rate of [2-\textsuperscript{14}C]propionate fixation</td>
<td>Rate of O\textsubscript{2} consumption\textsuperscript{a}</td>
</tr>
<tr>
<td>Propionate</td>
<td>23.9</td>
<td>45.1</td>
</tr>
<tr>
<td>Propionate+NaHCO\textsubscript{3}</td>
<td>34.5</td>
<td>59.9</td>
</tr>
<tr>
<td>Propionate+NaHCO\textsubscript{3}+itaconate, 1 mM</td>
<td>24.7</td>
<td>42.2</td>
</tr>
</tbody>
</table>

Cells were grown in the medium with propionate and bicarbonate (see Section 2). The concentrations of propionate and NaHCO\textsubscript{3} were 5 mM.

\textsuperscript{a}The rate of endogenous respiration was 25.3 nmol min\textsuperscript{−1}(mg protein)\textsuperscript{−1} for \textit{P. fulvum} and 14.9 nmol min\textsuperscript{−1}(mg protein)\textsuperscript{−1} for \textit{R. sphaeroides}.

The possible involvement of citramalate as an intermediate in bacterial primary metabolism has been emphasized by recent work of Herter \textit{et al.} [37]. These authors studied the fate of glyoxylate, which is synthesized during autotrophic CO\textsubscript{2} fixation by \textit{C. aurantiacus}. The proposed glyoxylate assimilation pathway is reversed to the citramalate cycle; it starts with glyoxylate and propionyl-CoA and affords acetyl-CoA and pyruvate with β-methylmalyl-CoA, mesaconyl-CoA, citramalate and citramalyl-CoA as intermediates.

3.4. Growth of \textit{R. rubrum} in the presence of itaconate

\textit{R. rubrum} grew on acetate or propionate in the presence of itaconate (0.1%; data not shown). Similar results were obtained for ICL\textsuperscript{+} methylotrophs which grew on C\textsubscript{1}\textsuperscript{−} and C\textsubscript{2}\textsuperscript{−} compounds in the presence of itaconate after a lag phase, in some cases with the same growth rates as control cultures [7]. Iaconate cannot serve as the sole carbon source for \textit{R. rubrum} (data not shown). Growth yields were about the same independently of the presence of itaconate even at very low (0.03%) levels of carbon substrate (acetate) in the medium. Moreover, itaconate did not stimulate oxygen consumption by the cells grown in the presence of itaconate (data not shown). These data suggest that \textit{R. rubrum} did not metabolize itaconate. In the case of \textit{R. rubrum} cells grown on acetate in the presence of itaconate, propionate and acetate fixation as well as respiration were not inhibited by itaconate (data not shown). PCC in cell extract of these cells, however, was still sensitive to itaconate (Table 2). The most probable explanation of these results is that the cells grown in the presence of itaconate are capable of excreting itaconate out of the cells or detoxifying it. We have not been able to test this hypothesis due to current unavailability of an expedient source of radiolabelled itaconate.

citramalate and lack of noticeable effect of this compound on cell respiration in the presence of acetate supports the idea of citramalate serving as an intermediate in acetate assimilation.


