Electronic Supplementary Information

α-Hydroxy-β-keto Acid Rearrangement-Decarboxylation: Impact on Thiamine Diphosphate-Dependent Enzymatic Transformations

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**Additional general experimental procedures:**

Sodium salts of [1,2-13C]- and [2-13C]-pyruvate were purchased from Sigma-Aldrich Co Ltd. Sodium salt of [1-13C]-pyruvate and L-[1,2-13C]-glutamic acid were purchased from Cambridge Isotope Labs.

The MenD gene from Escherichia coli K12 and yerE gene from Yersinia pseudotuberculosis were expressed and purified following standard molecular biology techniques as explained elsewhere.[1,2] The sucA gene of Escherichia coli K12 was cloned into a pET-28b (+) (Novagen) at NcoI and HindIII restriction sites. The synthetic gene of Azoarcus sp. 22LIN CDH (GeneArt, Invitrogen) was cloned into pET-21a vector (Novagen) at Ndel and Xhol restriction sides. The resulting constructs were full length sequenced to confirm in-frame cloning. The SucA and CDH recombinant proteins with C-terminal 6 x histidine tag were over-expressed in E. coli BL21(DE3) cells and were consequently purified following standard molecular biology techniques.[1]

For the coupled enzymatic system[3] the NADH oxidase from Lactobacillus brevis and L-glutamate dehydrogenase (L-GluDH) from Clostridium sp. were used.
$^{13}$C NMR studies with $\alpha$-KG and $^{13}$C-labeled pyruvate

\[
\begin{array}{c}
\text{MenD} \ [\text{Mg}^{2+}, \text{ThDP}]
\end{array}
\]

$\alpha$-KG + [1-$^{13}$C]-pyruvate

[1-$^{13}$C]-Pyruvate: $^{13}$C NMR (D$_2$O): $\delta = 170.1$ (s), 178.5 ppm (s, hydrate).

2-Hydroxy-2-methyl-3-oxohexanedioic acid (3): $^{13}$C NMR (D$_2$O): $\delta = 176.8$ ppm (s).

Carbonate: $^{13}$C NMR (D$_2$O): $\delta = 160.2$ ppm (s).

Carbon dioxide: $^{13}$C NMR (D$_2$O): $\delta = 124.6$ ppm (s).

$\alpha$-KG + [1,2-$^{13}$C]-pyruvate

[1,2-$^{13}$C]-Pyruvate: $^{13}$C NMR (D$_2$O): $\delta = 93.9$ (d, $J = 63$ Hz, hydrate), 170.2 (d, $J = 62$ Hz), 178.5 (d, $J = 63$ Hz, hydrate), 205.1 ppm (d, $J = 62$ Hz).

2-Hydroxy-2-methyl-3-oxohexanedioic acid (3): $^{13}$C NMR (D$_2$O): $\delta = 82.7$ (d, $J = 51$ Hz), 176.8 ppm (d, $J = 51$ Hz).

4-Hydroxy-5-oxohexanoic acid (2): $^{13}$C NMR (D$_2$O): $\delta = 214.8$ ppm (s).

Carbonate: $^{13}$C NMR (D$_2$O): $\delta = 160.2$ ppm (s).

Acetic acid as side product $^{13}$C NMR (D$_2$O): $\delta = 181.4$ ppm (s).

$\alpha$-KG + [2-$^{13}$C]-pyruvate

[2-$^{13}$C]-Pyruvate: $^{13}$C NMR (D$_2$O): $\delta = 93.8$ (s, hydrate), 205.1 ppm (s).

2-Hydroxy-2-methyl-3-oxohexanedioic acid (3): $^{13}$C NMR (D$_2$O): $\delta = 82.7$ ppm (s).

4-Hydroxy-5-oxohexanoic acid (2): $^{13}$C NMR (D$_2$O): $\delta = 214.8$ ppm (s).

Carbonate: $^{13}$C NMR (D$_2$O): $\delta = 160.2$ ppm (s).

Acetic acid as side product $^{13}$C NMR (D$_2$O): $\delta = 181.4$ ppm (s).
$^{13}$C NMR studies with α-KG and $^{13}$C-labeled acetaldehyde

\[
\begin{align*}
\text{HO} & -\text{CO}_2\text{H} + \text{CHO} \xrightarrow{\text{MenD} [\text{Mg}^{2+}, \text{ThDP}]} \text{HO} \xrightarrow{\text{L-GluDH}} \text{CO}_2\text{H} + \text{NH}_3 + \text{side reaction}
\end{align*}
\]

α-KG + [1,2-$^{13}$C]-acetaldehyde

[1, 2-$^{13}$C]Acetaldehyde: $^{13}$C NMR (D$_2$O): $\delta = 23.3$ (d, $J = 44$ Hz, hydrate), 30.2 (d, $J = 39$ Hz), 88.3 (d, $J = 44$ Hz, hydrate), 206.9 ppm (d, $J = 39$ Hz).

5-Hydroxy-4-oxohexanoic acid (1): $^{13}$C NMR (D$_2$O): $\delta = 18.5$ (d, $J = 36$ Hz), 72.8 ppm (d, $J = 36$ Hz).

$^{13}$C NMR kinetic studies with in situ production of $^{13}$C-labeled α-KG

L-[1,2-$^{13}$C]-Glutamic acid: $^{13}$C NMR (D$_2$O): $\delta = 54.8$ (d, $J = 53$ Hz), 174.6 ppm (d, $J = 53$ Hz).

L-[1,2-$^{13}$C]-Glutamic acid + [1-$^{13}$C]-pyruvate

2-Hydroxy-2-methyl-3-oxohexanedioic acid (3): $^{13}$C NMR (D$_2$O): $\delta = 176.8$ (s), 212.1 ppm (s).

4-Hydroxy-5-oxohexanoic acid (2): $^{13}$C NMR (D$_2$O): $\delta = 76.5$ ppm (s).
Carbonate: $^{13}$C NMR (D$_2$O): $\delta = 160.2$ ppm (s).

Carbon dioxide: $^{13}$C NMR (D$_2$O): $\delta = 124.6$ ppm (s).

Lactate: $^{13}$C NMR (D$_2$O): $\delta = 182.4$ ppm (s).

L-[1,2-$^{13}$C]-Glutamic acid + [1,2-$^{13}$C]-pyruvate

2-Hydroxy-2-methyl-3-oxohexanedioic acid (3): $^{13}$C NMR (D$_2$O): $\delta = 82.7$ (dd, $J = 51$, 42 Hz), 176.8 (d, $J = 51$ Hz), 212.0 ppm (d, $J = 42$ Hz).

4-Hydroxy-5-oxohexanoic acid (2): $^{13}$C NMR (D$_2$O): $\delta = 76.5$ (d, $J = 41$ Hz), 214.8 ppm (d, $J = 41$ Hz).

Carbonate: $^{13}$C NMR (D$_2$O): $\delta = 160.2$ ppm (s).

Carbon dioxide: $^{13}$C NMR (D$_2$O): $\delta = 124.6$ ppm (s).

Lactate: $^{13}$C NMR (D$_2$O): $\delta = 68.5$ (d, $J = 55$ Hz), 182.4 ppm (d, $J = 55$ Hz).

L-[1,2-$^{13}$C]-Glutamic acid + [2-$^{13}$C]-pyruvate

2-Hydroxy-2-methyl-3-oxohexanedioic acid (3): $^{13}$C NMR (D$_2$O): $\delta = 82.7$ (d, $J = 42$ Hz), 212.0 ppm (d, $J = 42$ Hz).

4-Hydroxy-5-oxohexanoic acid (2): $^{13}$C NMR (D$_2$O): $\delta = 76.5$ (d, $J = 41$ Hz), 214.8 ppm (d, $J = 41$ Hz).

Carbonate: $^{13}$C NMR (D$_2$O): $\delta = 160.2$ ppm (s).

Carbon dioxide: $^{13}$C NMR (D$_2$O): $\delta = 124.6$ ppm (s).

Lactate: $^{13}$C NMR (D$_2$O): $\delta = 68.5$ ppm (s).

Acetic acid as side product $^{13}$C NMR (D$_2$O): $\delta = 181.4$ ppm (s).
Summary table for the chemical shifts of $^{13}$C-labeled compounds

<table>
<thead>
<tr>
<th>MenD</th>
<th>Pyruvate</th>
<th>3</th>
<th>2</th>
<th>1</th>
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</thead>
<tbody>
<tr>
<td></td>
<td><img src="image" alt="Pyruvate Structure" /></td>
<td><img src="image" alt="3 Structure" /></td>
<td><img src="image" alt="2 Structure" /></td>
<td><img src="image" alt="1 Structure" /></td>
</tr>
<tr>
<td>C-1</td>
<td>ppm</td>
<td>ppm</td>
<td>ppm</td>
<td>ppm</td>
</tr>
<tr>
<td>C-2</td>
<td>ppm</td>
<td>ppm</td>
<td>ppm</td>
<td>ppm</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>ppm</th>
<th>ppm</th>
<th>ppm</th>
<th>ppm</th>
<th>ppm</th>
<th>ppm</th>
<th>ppm</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-KG + [1-$^{13}$C]-pyruvate</td>
<td>170.1(s)</td>
<td>-</td>
<td>176.8(s)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>α-KG + [1,2-$^{13}$C]-pyruvate</td>
<td>170.2(d)</td>
<td>205.1(d)</td>
<td>176.8(d)</td>
<td>82.7(d)</td>
<td>-</td>
<td>-</td>
<td>214.8(s)</td>
</tr>
<tr>
<td>α-KG + [2-$^{13}$C]-pyruvate</td>
<td>-</td>
<td>205.1(s)</td>
<td>-</td>
<td>82.7(s)</td>
<td>-</td>
<td>-</td>
<td>214.8(s)</td>
</tr>
<tr>
<td>α-KG + [1,2-$^{13}$C]-acetaldehyde</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>L-[1,2-$^{13}$C]-Glutamic acid + [1-$^{13}$C]-pyruvate</td>
<td>170.1(s)</td>
<td>-</td>
<td>176.8(s)</td>
<td>-</td>
<td>212.1(s)</td>
<td>76.5(s)</td>
<td>-</td>
</tr>
<tr>
<td>L-[1,2-$^{13}$C]-Glutamic acid + [1,2-$^{13}$C]-pyruvate</td>
<td>170.2(d)</td>
<td>205.1(d)</td>
<td>176.8(d)</td>
<td>82.7(dd)</td>
<td>212.1(d)</td>
<td>76.5(d)</td>
<td>214.8(d)</td>
</tr>
<tr>
<td>L-[1,2-$^{13}$C]-Glutamic acid + [2-$^{13}$C]-pyruvate</td>
<td>-</td>
<td>205.1(s)</td>
<td>-</td>
<td>82.7(d)</td>
<td>212.1(d)</td>
<td>76.5(d)</td>
<td>214.8(d)</td>
</tr>
</tbody>
</table>
Enzyme separation by ultrafiltration

To follow the intermediate 3 in the absence of the enzyme, the reaction was started with [1,2-\(^{13}\)C]-pyruvate and MenD under the standard reaction condition mentioned above (1.5 mL reaction volume). After 5 hours, the \(^{13}\)C-NMR experiment proved the formation of the intermediate. Then the enzyme was removed by ultrafiltration using Vivaspin 20 [10 MWCO] concentrator (Sartorius). Qualitative screening by Bradford assay in 96 well plates as well as SDS-PAGE confirmed complete removal of the protein from the solution. Then the solution was filled in NMR tube and the reaction was directly followed by sequential \(^{13}\)C-NMR experiments.

\(^{13}\)C NMR studies for YerE-catalyzed carboligation of pyruvate and 2-oxobutyrate:

\textbf{2-oxobutyrate + [2-\(^{13}\)C]-pyruvate}

2-oxobutyrate + [2-\(^{13}\)C]-pyruvate

\([2, 3-\(^{13}\)C]-acetolactate (4): \(^{13}\)C NMR (D\(_2\)O): \(\delta = 82.7\) (d, \(J = 43\) Hz), 211.7 ppm (d, \(J = 43\) Hz).

\([3-\(^{13}\)C]-acetoxybutyrate (5): \(^{13}\)C NMR (D\(_2\)O): \(\delta = 212.0\) ppm (s).

\([1, 2-\(^{13}\)C]-acetoin (6): \(^{13}\)C NMR (D\(_2\)O): \(\delta = 73.1\) (d, \(J = 41\) Hz), 215.5 ppm (d, \(J = 41\) Hz).

\([3-\(^{13}\)C]-acetohydroxybutyrate (5): \(^{13}\)C NMR (D\(_2\)O): \(\delta = 212.0\) ppm (s).

\([1, 2-\(^{13}\)C]-acetoin (6): \(^{13}\)C NMR (D\(_2\)O): \(\delta = 73.1\) (d, \(J = 41\) Hz), 215.5 ppm (d, \(J = 41\) Hz).

\([1, 2-\(^{13}\)C]-acetoin (6): \(^{13}\)C NMR (D\(_2\)O): \(\delta = 73.1\) (d, \(J = 41\) Hz), 215.5 ppm (d, \(J = 41\) Hz).

References:


CD spectra:

MenD-catalyzed reaction of α-KG with (a) pyruvate or (b) acetaldehyde over time. Time delay between each scan was set to 60 minutes.
NMR spectra: α-KG + [1-\textsuperscript{13}C]-pyruvate

after 24h

after 48h
α-KG + [1,2-\textsuperscript{13}C]-pyruvate

After 5h

After 48h

ppm (11)
\[ \alpha\text{-KG} + [2-^{13}\text{C}]\text{-pyruvate} \]
$\alpha$-KG + [1,2-$^{13}$C]-acetaldehyde

After 3h

After 12h
L-[1,2-\textsuperscript{13}C]-Glutamic acid + [1-\textsuperscript{13}C]-pyruvate

After 3h

After 24h
$\text{L-}[1,2^{-13}\text{C}]\text{-Glutamic acid} + [1,2^{-13}\text{C}]\text{-pyruvate}$
**L-[1,2-\text{\textsuperscript{13}}C]-Glutamic acid + [2-\text{\textsuperscript{13}}C]-pyruvate**

After 8h

**After 24h**
2-Oxobutyrate + [2-\textsuperscript{13}C]-pyruvate

After 2h

After 40h
Spectra of Deuterium labeling experiments

\(^1\)H NMR of 1 in CD\(_3\)OD (up)
\(^2\)H NMR of 1 in CH\(_3\)OH after incubation in D\(_2\)O for 48 h (bottom)

\(^{13}\)C NMR of 1 in CD\(_3\)OD after incubation in D\(_2\)O for 48 h