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α-Hydroxy-β-keto acid rearrangement–decarboxylation: impact on thiamine diphosphate-dependent enzymatic transformations†

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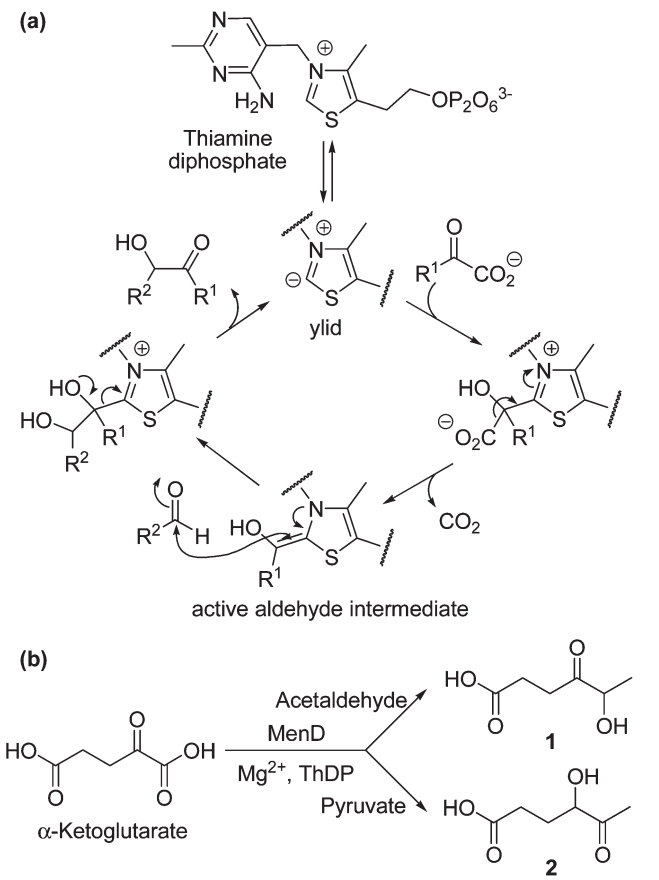
The thiamine diphosphate (ThDP) dependent MenD catalyzes the reaction of α-ketoglutarate with pyruvate to selectively form 4-hydroxy-5-oxohexanoic acid 2, which seems to be inconsistent with the assumed acyl donor role of the physiological substrate α-KG. In contrast the reaction of α-ketoglutarate with acetaldehyde gives exclusively the expected 5-hydroxy-4-oxo regioisomer 1. These reactions were studied by NMR and CD spectroscopy, which revealed that with pyruvate the observed regioselectivity is due to the rearrangement–decarboxylation of the initially formed α-hydroxy-β-keto acid rather than a donor–acceptor substrate role variation. Further experiments with other ThDP-dependent enzymes, YerE, SucA, and CDH, verified that this degenerate decarboxylation can be linked to the reduced enantioselectivity of acyloins often observed in ThDP-dependent enzymatic transformations.

Introduction

Thiamine diphosphate (ThDP)-dependent enzymes participate in numerous biosynthetic pathways and catalyze a wide range of reactions mainly involved in C–C bond formation or cleavage adjacent to a carbonyl group.[1,2] One of the basic model reactions of this enzyme family is the decarboxylation of an α-keto acid (the acyl donor) to give the active aldehyde intermediate which adds to an aldehyde (the acyl acceptor) to form the corresponding α-hydroxy ketone (an acyloin), Scheme 1a. This type of reaction is synthetically attractive as formation of C–C bonds in this way is not easy and enzymes have the potential of producing chiral products with high enantiomeric excess (ee).

MenD (2-succinyl-5-enolpyruvyl-6-hydroxy-3-cyclohexene-1-carboxylate synthase) from *E. coli* is a ThDP-dependent enzyme involved in menaquinone biosynthesis which uses α-ketoglutarate (α-KG) as the natural acyl donor and



Scheme 1 (a) Mechanism of the ThDP-dependent formation of acyloins. (b) Two regioisomeric acyloins are formed by MenD from α-KG depending on the second substrate.

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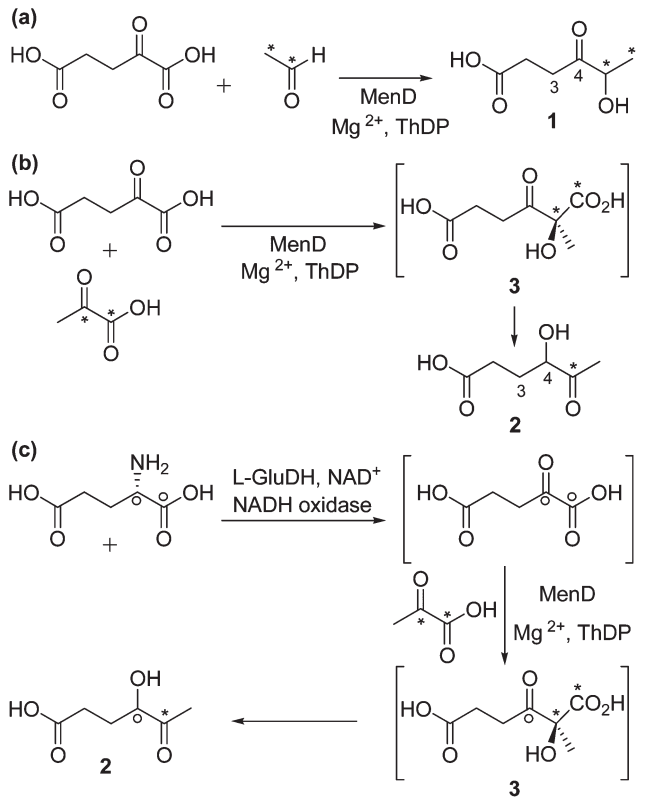
isochorismate as the acceptor.[3–5] We have previously reported that acyloin **1** was formed as the sole product when acetaldehyde was incubated with α-KG and MenD, whereas the use of pyruvate instead of acetaldehyde led exclusively to the formation of **2**, an apparent reversal of the donor–acceptor roles of the two substrates (Scheme 1b).[5] However, the donor substrate spectrum of MenD is known to be narrow and pyruvate is accepted as a donor only in trace amounts by MenD when α-KG is absent.[5,6] Therefore, we reasoned that the regioselectivity might not be due to a switch in the donor substrate, as it appears at first sight, but instead to a selective rearrangement–decarboxylation of an initially formed α-hydroxy-β-keto acid product. Here we report on experiments using ^{13}C-labeled substrates to elucidate the mechanism of the selective formation of **2**, and the consequences for similar enzymatic and nonenzymatic transformations.

Results and discussion

Incubation of α-KG with [1,2-$^{13}C_2$]-acetaldehyde and MenD led, as expected, to the formation of **1** as the sole product, with the ^{13}C labels exclusively in the CH_3CHOH group (Scheme 2a). No product **2** was observed.

The use of α-KG with [2-^{13}C] or [1,2-$^{13}C_2$]pyruvate and following the reaction by ^{13}C NMR spectroscopy showed that the first formed intermediate is 2-hydroxy-2-methyl-3-oxohexanedioic acid **3** (Scheme 2b). This would be the expected product if α-KG is the acyl donor and pyruvate the acceptor. On continued monitoring of the incubation mixture, the intensity of the signals for **3** decreased over time while signals for **2** increased and became dominant (see ESI†). Extraction after complete consumption of the reactants gave nearly racemic **2** as the main product, in agreement with published data.[5]



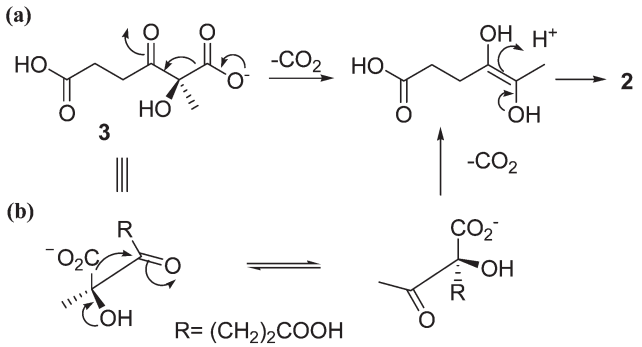
Scheme 2 MenD-catalyzed C–C bond forming reactions with ^{13}C-labeled substrates. In (c) [1,2-$^{13}C_2$]α-KG was made by *in situ* enzymatic oxidation of L-glutamate. The labeled C atoms are marked with circles and asterisks.

In order to investigate whether the observed decarboxylation occurs through any detectable intermediate, ^{13}C-labeled α-KG was synthesized by oxidation of ^{13}C-labeled L-glutamate using L-glutamate dehydrogenase (L-GluDH) from *Clostridium* sp., coupled with NADH oxidase from *Lactobacillus brevis*[7] for simultaneous regeneration of the cofactor.[8] Biotransformation of the *in situ* formed [1,2-$^{13}C_2$]α-KG with MenD and pyruvate was then followed by ^{13}C NMR spectroscopy. As expected, L-glutamate was fully consumed by the coupled enzyme system; however, some reduction of pyruvate to lactate was observed as a side reaction. Hence, pyruvate was added in excess to avoid substrate limitation.

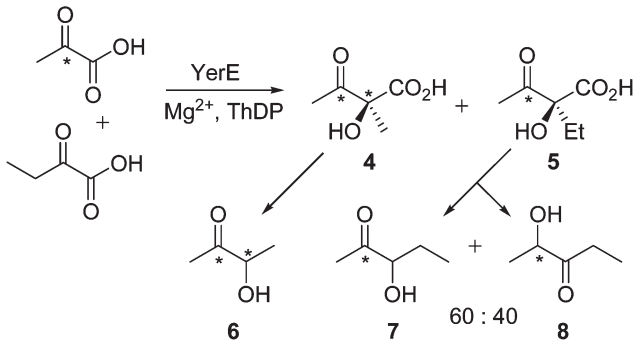
Repetition of the NMR experiments using both L-[1,2-$^{13}C_2$] glutamate and [1-^{13}C], [2-^{13}C] or [1,2-$^{13}C_2$]pyruvate again demonstrated formation of **3** as the first observed intermediate, which eventually underwent decarboxylation to give **2** as the sole final product (Scheme 2c). Sequential ^{13}C NMR measurements were performed every hour for 48 hours. No intermediate between **3** and **2** could be detected in these experiments. It is worth noting that the observed regiochemistry of the decarboxylation product is unlikely to be of thermodynamic origin as incubation of α-KG with acetaldehyde in the presence of MenD leads to the formation of the isomeric **1** as the sole product without any detectable traces of **2**. Upon further incubation in D_2O for 48 hours, **2** proved to be unstable. In contrast, **1** was quite stable and the only deuterium incorporation observed was at C-3 (see ESI†).

Analysis by *in situ* CD spectroscopy[9] indicated that the (*S*)-enantiomer of **3** had been formed, as indicated by the appearance of a positive CD band at 300 nm. No CD signal was observed for **1** or **2** indicating that both products were virtually racemic (see ESI†). This was confirmed by chiral phase LC-MS.

The observation that the decarboxylation of **3** produces specifically **2** initially suggested that a rearrangement–decarboxylation, as has been proposed for the decarboxylation of (*R*)-acetolactate by the acetolactate decarboxylase from *Klebsiella aerogenes*,[10,11] might also be occurring enzymatically in our case. Thus, two possible mechanisms are as shown in Scheme 3, in which decarboxylation, with or without carboxyl group migration, gives the enediol intermediate, which could be regioselectively protonated by the enzyme to give only **2**. However, the racemic nature of **2** would be highly unusual for an enzymatic product. Furthermore, separation of the enzyme by ultrafiltration (for details, see ESI†) after the formation of the intermediate **3** still led to formation of only **2** with no trace of **1**. Therefore, the observed decarboxylation is probably nonenzymatic. However, some impact of the protein on this transformation cannot be excluded as the nonenzymatic reaction



Scheme 3 Possible pathways for the decarboxylation of **3** *via* (a) direct decarboxylation or (b) tertiary ketol rearrangement, followed by decarboxylation.



Scheme 4 YerE-catalyzed formation of acetohydroxy acids **4** and **5**, and subsequent decarboxylation to acyloins **6–8**. The labeled C atoms are marked with an asterisk.

never proceeded to completion, whereas it did in the presence of MenD.

If the decarboxylation is nonenzymatic, this leaves the question of why the protonation of the enediol intermediate is regiospecific, forming only **2**. The probable explanation is related to the presence of the carboxylic acid functionality in **3**, which could be a general acid catalyst and would be expected to protonate C-4 much more efficiently than C-5, due to the more favorable ring size. In support of this, decarboxylation of **3**, after the removal of enzyme, at alkaline pH (pH 13, to ensure the carboxylic acid is fully deprotonated) was found to produce a mixture of **1** and **2**. Unfortunately, further experiments to test these hypotheses using other donor substrates, such as 2-oxoglutarate 5-monoethyl ester or oxaloacetate (a nonphysiological donor known to be accepted by MenD in the presence of 2-fluorobenzaldehyde[5]), failed since these donors were not accepted as substrates by MenD with pyruvate as the acceptor.

α-Keto acids are frequently accepted as acyl acceptor substrates in reactions catalyzed by different ThDP-dependent enzymes. Therefore, α-hydroxy-β-keto acids are often formed and similar decarboxylation to that described above can be expected. In order to test whether this is generally the case, three further ThDP-dependent enzymes were investigated.

First [2-^{13}C]pyruvate was incubated with 2-oxobutyrate in the presence of ThDP-dependent YerE.[12] This transformation initially led to the formation of (*S*)-acetolactate (**4**) (from two molecules of pyruvate) and (*S*)-acetohydroxybutyrate (**5**) (from pyruvate as the donor and 2-oxobutyrate as the acceptor) (Scheme 4).[13] The NMR data showed that 2-oxobutyrate is the acceptor preferred by the enzyme (ratio **5** : **4** = 84 : 16 after 17 h). When this reaction was followed for a longer time, it was found that besides acetoin (**6**), acyloin regioisomers **7** and **8** were also formed in a 60 : 40 ratio as the final products (Scheme 4). Acyloin **8** can only be derived by decarboxylation of **5**, since the possibility of 2-oxobutyrate being the acyl donor was excluded by further ^{13}C NMR experiments using [1,2-$^{13}C_2$] pyruvate.

SucA, the ThDP-dependent E1 subunit of the α-ketoglutarate dehydrogenase complex from *E. coli* K12,[14] was studied next. Incubation of α-KG and labeled pyruvate with SucA led to similar results regarding regioselectivity to those observed with MenD. Additionally, SucA catalyzed the formation of acetoin using either pyruvate or acetaldehyde as the sole substrate. Incubation of pyruvate with SucA led, *via* **4**, to almost racemic acetoin **6** with only 8% ee of the (*R*)-isomer but the ee increased to 90% when acetoin was prepared using acetaldehyde as the substrate.

In contrast, NMR investigations with ThDP-dependent CDH (cyclohexanedione hydrolase from *Azoarcus* sp.)[15] showed no formation of acetolactate as an intermediate, starting either from [1,2-^{13}C]pyruvate as the sole substrate or in combination with acetaldehyde. Accordingly, highly enantio-enriched (*S*)-acetoin [(*S*)-**6**] (up to 90% ee) was directly obtained using pyruvate as the sole substrate. This can be explained by enzymatic decarboxylation of pyruvate to give acetaldehyde, which then acts as the acyl acceptor substrate giving (*S*)-acetoin.

Conclusions

From the results with MenD, YerE and SucA, it can be seen that the formation and subsequent decarboxylation of α-hydroxy-β-keto acids is common when α-keto acids are involved as substrates in ThDP-dependent enzymatic transformations. As shown above, this often leads to the formation of the mixed regioisomers of the corresponding α-hydroxy ketones, but also might lead to the selective formation of a sole product, as in the decarboxylation of **3**. We postulate that the decarboxylation of optically active acetolactate is the reason for the formation of the nearly racemic acetoin observed in many ThDP-dependent enzymatic transformations.[16]

In conclusion, the change in regioselectivity induced by varying the substrate(s) in ThDP-dependent enzymatic transformations may not necessarily be due to swapping the acyl donor–acceptor roles of the substrates. The observation of unexpected products, such as **2**, which initially appear to be the result of acceptor–donor reversal, can lead to misinterpretation and incorrect characterization of an enzyme. Understanding the selective formation of **2** by MenD paved the way

for the discovery that CDH produces highly enantioenriched acetoin from pyruvate as the sole substrate, *because* it is incapable of producing acetolactate.

Experimental

General procedures

Nuclear magnetic resonance (NMR) spectra were recorded on a DRX 400 (*Bruker*) operating at 400 and 100 MHz for ^{1}H and ^{13}C acquisitions, respectively. Coupling constants (*J*) are reported in Hertz (Hz). Circular dichroism (CD) was measured using a spectral polarimeter J-810 (*Jasco International*). High-performance liquid chromatography (HPLC-DAD) was performed on an HP 1100 chromatography system (*Agilent*). HPLC-DAD with MS/MS (LC-MS) was performed using API2000 with a TurbolonSpray™ source, EI, MRM scan (*Applied Biosystems*). LC on a chiral phase was performed on an Astec Chirobiotic T 5 μm column, at 5 °C, eluted at 0.3 mL min^{-1} with methanol, 0.1% acetic acid and 0.4% triethylamine. GC on a chiral phase was performed on GC-2010 (FID) at 70 °C equipped with the injector AOC-20 (*Shimadzu*) using a Macherey-Nagel, FS-Lipodex D column (50.0 m × 0.25 mm).

Other general procedures are given in the ESI.†

General procedure for formation of 1 or 2

MenD or SucA (500 μg mL^{-1}) and α-KG (30 mM) was incubated with pyruvate or acetaldehyde (30 mM) in a reaction buffer (1.5 mL; 50 mM phosphate, 2 mM $MgCl_2$, 0.1 mM ThDP, 10% (v/v) D_2O, pH = 8) at 30 °C and 300 rpm using a thermomixer (Eppendorf). After 48 h, the sample was used for NMR without any further purification.

For *in situ* ^{13}C NMR experiments, 15 mM of each substrate was incubated in 750 μL of the reaction buffer directly in an NMR tube under the same conditions as above. For selected kinetic experiments, the time delay between spectra was 60 min.

For *in situ* CD experiments,[9] 15 mM of each substrate was incubated in the reaction buffer in a 0.1 cm path length cell at 30 °C. The spectra were first recorded with the buffer to make sure that there is no background signal. The reactions were initiated by addition of the enzyme and the spectra were recorded in the range of 240–350 nm. The time delay between spectra was 60 min.

5-Hydroxy-4-oxohexanoic acid **1**, δ_H (D_2O) 1.26 (3 H, d, *J* 7.1), 2.27–2.32 (2 H, m), 2.65–2.70 (2 H, m) and 4.32 (1 H, q, *J* 7.1); δ_C (D_2O) 18.4 (CH_3), 30.8 (CH_2), 34.1 (CH_2), 72.8 (CH), 181.3 (COOH) and 215.8 (C═O); Chiral LC-MS: $t_R(R)$ = 6.8 min, $t_R(S)$ = 6.2 min; MS/MS: 145 (parent ion, $M - H^+$), 127, 101, and 83 (fragment ions).

4-Hydroxy-5-oxohexanoic acid **2**, δ_H (D_2O) 1.67–1.76 (2 H, m), 1.96–2.05 (2 H, m), 2.14 (3 H, s), 4.22 (1 H, dd, *J* 8.3, 3.9); δ_C (D_2O) 25.3 (CH_3), 28.9 (CH_2), 29.0 (CH_2), 76.4 (CH), 182.0 (COOH), 214.8 (C═O); Chiral LC-MS: $t_R(R)$ = 16.2 min, $t_R(S)$ = 17.3 min; MS/MS: 145 (parent ion, $M - H^+$), 127, 101, and 83 (fragment ions).

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

MenD (500 μg mL^{-1}), L-GluDH (4 U mL^{-1}), NADH oxidase (25 U mL^{-1}), L-[1,2-^{13}C]glutamic acid (30 mM), NAD^+ (5 mM) and DTT (5 mM) were incubated with ^{13}C-labeled pyruvate (30 mM) in the reaction buffer (750 μL) at 30 °C directly in an NMR tube. The reactions were followed by *in situ* NMR experiments.

Deuterium labeling experiments

The general procedure for formation of **1** or **2** was followed. Formation of **1** and **2** was confirmed by NMR and the reaction mixtures were then lyophilized. The solid residues were dissolved in D_2O (1.5 mL) and were incubated for a further 48 hours.

For **1**: δ_D (CH_3OH) 2.8 (br s); δ_C (CD_3OD) 20.0 (CH_3), 32.6 (CH_2), 35.5 (br, CH_2), 74.2 (CH), 181.2 (COOH), 216.1 (br, C═O).

^{13}C NMR studies of YerE-catalyzed carboligation of pyruvate and 2-oxobutyrate

YerE (1 mg mL^{-1}) and 2-oxobutyrate (50 mM) were incubated with [1,2-^{13}C] or [2-^{13}C]pyruvate (50 mM) in the reaction buffer (750 μL) at 25 °C directly in an NMR tube. ^{13}C NMR spectra were recorded with a time delay between spectra of 30 min.

SucA-catalyzed acetoin formation

SucA (700 μg mL^{-1}) and pyruvate or acetaldehyde (50 mM) were incubated in the reaction buffer (1.5 mL) at 30 °C and 300 rpm using a thermomixer (Eppendorf). After 48 h, the sample was used for NMR and GC measurements without any further purification. Chiral GC: $t_R(R)$ = 17.6 min, $t_R(S)$ = 13.2 min.

CDH-catalyzed acetoin formation

CDH (1 mg mL^{-1}) and pyruvate (25 mM) were incubated in the reaction buffer (1.5 mL; 50 mM MES, 3 mM $MgSO_4$, 0.5 mM ThDP, 10% (v/v) D_2O, pH = 6.5) at 30 °C and 300 rpm using a thermomixer (Eppendorf). After 24 and 48 h, the sample was used for GC measurements without any further purification.

For *in situ* ^{13}C-NMR, [1,2-$^{13}C_2$]pyruvate (25 mM) was incubated in the reaction buffer (750 μL) directly in an NMR tube under the same conditions as above. The time delay between spectra was 30 min.

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