

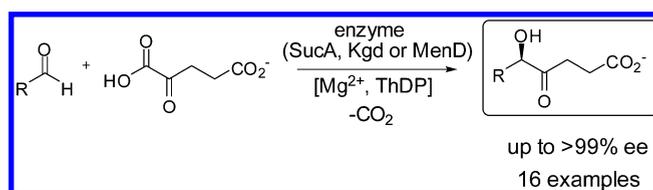
TCA Cycle Involved Enzymes SucA and Kgd, as well as MenD: Efficient Biocatalysts for Asymmetric C—C Bond Formation

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ABSTRACT



Asymmetric mixed carbonylation reactions of α -ketoglutarate with different aldehydes were explored with the thiamine diphosphate dependent enzymes SucA from *E. coli*, Kgd from *Mycobacterium tuberculosis*, and MenD from *E. coli*. All three enzymes proved to be efficient biocatalysts to selectively deliver chiral δ -hydroxy- γ -keto acids with moderate to excellent stereoselectivity. The high regioselectivity is due to the preserved role of α -ketoglutarate as acyl donor for these enzyme-catalyzed reactions.

The tricarboxylic acid (TCA) cycle is a key component of the central metabolism in aerobic organisms.¹ Within the TCA cycle, carbohydrates, fats, and proteins are catabolized into carbon dioxide and generate NADH, which subsequently promotes oxidative phosphorylation to provide ATP as the source of usable energy. In the anabolic function of the cycle, α -ketoglutarate (α -KG) is produced as a precursor of glutamate, which is subsequently converted into succinate (often through succinyl CoA). The oxidative decarboxylation of α -KG to succinyl CoA is known to be catalyzed by the α -ketoglutarate dehydrogenase (KDH) enzyme complex.² In principle, KDH is similar to pyruvate dehydrogenase as it is composed of the following three enzymes: thiamine diphosphate (ThDP) dependent 2-oxo acid decarboxylase (E1), lipoate-dependent acyl

transferase (E2), and FAD-dependent dihydrolipoyl dehydrogenase (E3).³

The function of KDH starts with the decarboxylation of α -KG by the E1 subunit, also designated as α -ketoglutarate decarboxylase, to form the ThDP adduct of succinyl semialdehyde (SSA). ThDP-dependent enzymes are known to be involved in diverse ligase and lyase reactions.⁴ The ThDP-dependent enzyme MenD (2-succinyl-5-enolpyruvyl-6-hydroxy-3-cyclohexene-1-carboxylate synthase) from *E. coli* K12 is a versatile biocatalyst for asymmetric synthesis.⁵ MenD is involved in the second step of menaquinone biosynthesis and catalyzes a Stetter-like 1,4-addition of α -KG to isochorismate.^{5d} Like MenD, the E1 subunit of

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KDH also accepts α -KG as the physiological (donor) substrate.

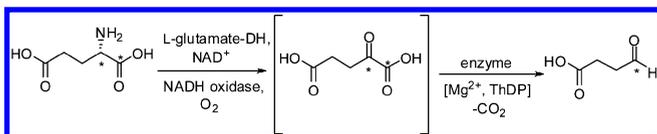
In a continuation of our studies on MenD,^{5d} we were interested in investigating whether SucA and Kgd,⁶ which are known for their decarboxylation activity, can also catalyze asymmetric C–C bond formations. In the presence of an appropriate acceptor, chiral δ -hydroxy- γ -keto acids can be synthesized as products. These are potent precursors of γ - and δ -lactones which are present in the structure of several natural products and are important intermediates in organic synthesis.^{7,8} In this study, we focused on mixed carbonylation reactions of two different carbonyl compounds using α -KG as one substrate.

The *sucA* gene in *E. coli* K12⁹ encodes the E1 subunit of KDH. It is a homologue of the *odhA* gene in *Corynebacterium glutamicum*¹⁰ and *Bacillus subtilis*.¹¹ In *Mycobacterium tuberculosis*, the homologous protein is Kgd which carries additional E2 sequences, as is also the case in *C. glutamicum*. The Kgd protein is essential for cell survival of *M. tuberculosis*, but an additional KG:ferredoxin oxidoreductase may bypass the requirement for KDH activity under anaerobic conditions.^{12,13}

The SucA and Kgd recombinant proteins were designed with a C-terminal hexa-histidine tag and were overexpressed in *E. coli* BL21(DE3) cells. Enzymes were purified by immobilized metal chelate chromatography (Ni-NTA). Production of MenD was performed as described previously.^{5d}

In order to verify the decarboxylase activity of these enzymes, the release of SSA from a solution of α -KG and enzyme was followed. ¹³C-Labeled α -KG was synthesized by oxidation of [1,2-¹³C]-L-glutamate using L-glutamate dehydrogenase (L-glutamate-DH) from *Clostridium* sp. coupled with NADH oxidase from *Lactobacillus brevis*.¹⁴ In situ biotransformation of the produced [1,2-¹³C]- α -KG with each of the three enzymes (MenD, SucA and Kgd) was then followed by ¹³C NMR spectroscopy (Scheme 1).

Scheme 1. Synthesis and Use of ¹³C-Labeled α -KG by Enzymatic Oxidation of L-Glutamate Coupled with In Situ Decarboxylation^a



^aThe ¹³C-labeled C atoms are marked with an asterisk.

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Furthermore, the 2,4-dinitrophenylhydrazones of the reaction compounds were prepared at the starting point, and at the end of the reaction, and were analyzed by HPLC. NMR and HPLC assays both showed that the incubation of α -KG in the presence of SucA and Kgd led to the release of SSA in solution,¹² while we were unable to detect any SSA in the case of MenD. Nevertheless, the release of CO₂ was confirmed in the case of all three enzymes with NMR experiments (using ¹³C-labeled α -KG).¹⁵

Next, the catalytic activity of all three enzymes with various aldehydes using the physiological acyl donor α -KG was investigated. The reaction conditions were optimized for each enzyme using 2-fluorobenzaldehyde and α -KG as a model reaction (see the Supporting Information). All reactions were performed on an analytical scale (1.5 mL reaction volume), and at least one example of each reaction type was undertaken on a semipreparative scale (15 mL reaction volume, 0.2 mmol acceptor substrate).

As indicated by the aromatic substrates, all three enzymes accepted a broad range of substituted benzaldehydes (Table 1, products 1–8). The electronic features of the acceptor substrate have a direct impact on the reaction, as the presence of an electron-withdrawing group such as halide led to improved results while the presence of an electron-donating group such as methoxy led to modest results, except for the cases with MenD. The presence of a nitro substituent at the *ortho* position resulted in no product formation for all three enzymes and therefore is not shown in Table 1. Substitution at different positions of the aromatic ring does not seem to play a significant role based on the results with chlorobenzaldehydes. SucA and Kgd showed lower conversion and significantly decreased enantioselectivity compared to MenD. As a result, MenD is the preferred enzyme when aromatic acceptor aldehydes are applied, resulting in *R*-configured products with good to excellent regio- and enantioselectivity (76 to >99% ee (enantiomeric excess)).

Further substrate studies were performed with a series of aliphatic aldehydes with different chain length (Table 1, products 9–12). In contrast to the aromatic aldehydes, here, SucA and Kgd delivered better results than MenD, especially in terms of enantioselectivity. In the case of SucA, enantioselectivity dropped significantly when sterically demanding aldehydes were used, whereas reverse results were obtained with MenD as the catalyst (<63% ee). In the case of Kgd, there was no indication of an impact of substrate steric demand on the ee. Overall, all three

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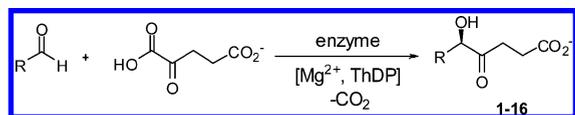
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(15) These observations with purified MenD are in contrast to those previously reported using supernatant enzyme (ref 5b).

Table 1. Conversion and ee Values for the Enzyme-Catalyzed 1,2-Addition of α -KG to Various Aldehydes



R	product	conversion ^a (%) (ee ^b (%)) of enzyme		
		SucA	Kgd	MenD
Ph	1	5 (6)	9 (17)	>99 (99)
2-FC ₆ H ₄	2	50 (31)	45 (26)	>99 (94)
2-ClC ₆ H ₄	3	61 (41)	50 (45)	98 (93)
2-BrC ₆ H ₄	4	43 (57)	57 (65)	82 (80)
2-IC ₆ H ₄	5	5 (nd)	50 (68)	90 (76)
2-MeOC ₆ H ₄	6	35 (10)	2 (nd)	98 (98)
3-ClC ₆ H ₄	7	50 (43)	20 (52)	>99 (96)
4-ClC ₆ H ₄	8	45 (3)	25 (20)	>99 (93)
CH ₃	9^c	>99 (94)	>99 (76)	>99 (<5)
CH ₃ CH ₂	10^c	>99 (94)	>99 (82)	>99 (11)
CH ₃ (CH ₂) ₃	11^c	>99 (90)	>99 (70)	>99 (63)
CH ₃ (CH ₂) ₄	12^c	>99 (82)	>99 (72)	>99 (61)
PhCH=CH	13	8 (nd)	nc ^d	89 (nd)
PhCH=C(CH ₃)	14	4 (nd)	nc	>99 (>99)
CH ₃ CH ₂ CH=CH	15	nc	nc	>99 (nd)
CH ₃ CH ₂ CH=C(CH ₃)	16	12 (nd)	nc	98 (nd)

^a Determined by NMR spectroscopy. ^b The absolute configuration for all products was determined as (*R*) on the basis of circular dichroism. ^c The conversion was determined to be quantitative as no aldehyde was detected in the crude mixtures by NMR or GC-MS analysis. ^d nc: no conversion; nd: not determined.

enzymes gave excellent conversion for the formation of δ -hydroxy- γ -keto acids from aliphatic aldehydes. SucA and Kgd gave the expected products with moderate to good enantioselectivity (70–94% ee).

Next, the potential of different α,β -unsaturated aldehydes to act as acceptor substrates was evaluated. For this type of substrate, addition of the decarboxylated α -KG as the acyl donor can occur either at the carbonyl moiety in a 1,2-addition or at the conjugated double bond, which forms the Stetter product.¹⁶ As shown in Table 1 (products **13–16**), Kgd and SucA led to no conversion or very poor results while MenD gave the desired products (1,2-addition) in excellent regio- and enantioselectivity. The formation of 1,4-addition products was not observed.

It is noteworthy to mention that products **13** and **14** can be considered as potential precursors of the styrenyl δ -lactones which are observed in several bioactive compounds, for example 5-hydroxygoniothalamin (Scheme 2).^{7b}

In order to determine the acyl donor spectrum of all three enzymes, different α -keto acids were tested as substrates with benzaldehyde or 2-fluorobenzaldehyde as acceptor. As shown in Table 2, MenD and Kgd are very specific with respect to the donor substrate as they poorly

Scheme 2. Lactonization of γ -Keto Acid **13** to the Corresponding δ -Lactone as a Possible Precursor for the Anticancer Compound 5-Hydroxygoniothalamin

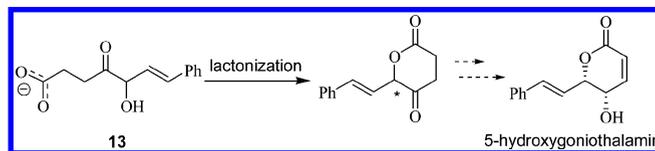
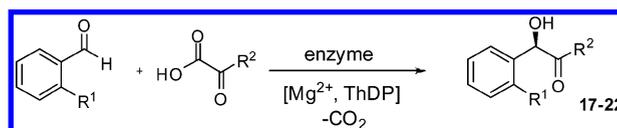


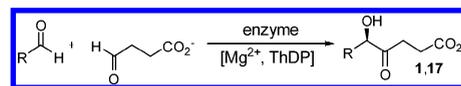
Table 2. Donor Substrate Spectra for the Enzyme-Catalyzed 1,2-Addition of Various α -Keto Acids to Aromatic Aldehydes



R ¹	R ²	product	conversion ^a (%) (ee ^b (%)) of enzyme		
			SucA	Kgd	MenD
H	CH ₃	17	26 (78)	nc ^c	nc
F	CH ₃	18	37 (16)	nc	5 (nd)
H	CH ₂ CH ₃	19	32 (94)	nc	nc
F	CH ₂ CH ₃	20	52 (60)	6 (nd)	nc
F	CH ₂ COOH	21	nc	nc	20 (nd)
F	(CH ₂) ₃ COOH	22	nc	nc	nc

^a Determined by GC-MS. ^b Determined by chiral-phase HPLC (see the Supporting Information). ^c nc: no conversion; nd: not determined.

Table 3. Conversion and ee Values for the Enzyme-Catalyzed 1,2-Addition of SSA to an Aromatic or Aliphatic Aldehyde



R	product	enzyme		
		SucA	Kgd	MenD
conversion ^a (%), (ee ^b (%))				
CH ₃	17	quant (76)	nc ^c	20 (<5)
Ph	1	1 (nd)	nc	11 (99)

^a Determined by NMR spectroscopy for **17** and by GC-MS for **1**. ^b The absolute configuration for all products was determined as (*R*) on the basis of circular dichroism. ^c nc: no conversion; nd: not determined.

accept any other α -keto acid. In contrast, SucA showed a broader donor substrate spectrum.

Furthermore, SSA was tested as a possible acyl donor, and the results are shown in Table 3. Interestingly, SucA and MenD showed a tolerance to accept SSA, whereas the use of Kgd led to no product formation, independent of the acceptor type.

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Table 4. Carboligation Efficiency of SucA and the H460I Variant

product	conversion ^a (%) (ee ^b (%))	
	SucA	SucA-H460I
1	7 (6)	94 (93)
2	52 (31)	99 (83)

^aDetermined by GC–MS. ^bThe absolute configuration for all products was determined as (*R*) on the basis of circular dichroism.

In summary, we have characterized the substrate spectra of enzymes from the central metabolism of aerobic organisms, namely SucA from *E. coli* and Kgd from *M. tuberculosis*, in comparison with that of MenD from *E. coli*. It was shown that these three enzymes feature a broad, but different, substrate specificity for cross-acyloin condensations. It was demonstrated that a wide range of substrates, including aliphatic, aromatic and α,β -unsaturated aldehydes, in combination with α -KG can be used, resulting in the expected δ -hydroxy- γ -keto acids.

The products are formed with moderate to excellent enantioselectivity. In order to enhance the efficiency of these selected enzymes, protein engineering can be applied to prepare variants with improved carboligation efficiency or showing *S*-selectivity.¹⁷ To identify the critical residues responsible for the differences in the activity, we compared the structure of MenD (PDB entry 3HWX) with the structure of Kgd from *Mycobacterium smegmatis* (MsKgd, PDB entry 2YID). The overlay of the active sites

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revealed a hydrophobic pocket of MenD which is interrupted by a histidine in MsKgd, a probable hotspot. Then MsKgd and SucA were aligned on the basis of the primary sequence. The resulting variant SucA-H460I (Fries and Müller, to be published) led to excellent conversions and enantioselectivities using aromatic substrates (Table 4).

Chemoselectivity, which is often a challenge in mixed carboligation reactions,¹⁸ was excellent, independent of the substrate or enzyme used. This is due to the preserved role of α -KG as donor with these enzymes. Furthermore, self-condensation of α -KG was never observed.

In addition, we have demonstrated that succinic semi-aldehyde, even as a direct substrate, can be successfully applied as a donor using ThDP-dependent enzymes. So far, acetaldehyde (pyruvate), glyoxal (hydroxypyruvate), and aliphatic aldehydes have been shown to be the preferred donor substrates for ThDP-dependent enzymes.^{4b} Therefore, our results expand the range of substituted acetaldehydes (glyoxal, and methoxy- and dimethoxyacetaldehyde) as substrates toward C4-functionalized aldehydes.

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Supporting Information Available. Detailed experimental procedures and characterization data of all new compounds. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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The authors declare no competing financial interest.