ChemPubSoc Europe

DOI: 10.1002/cbic.201300673

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Catalytic Scope of the Thiamine-Dependent Multifunctional Enzyme Cyclohexane-1,2-dione Hydrolase

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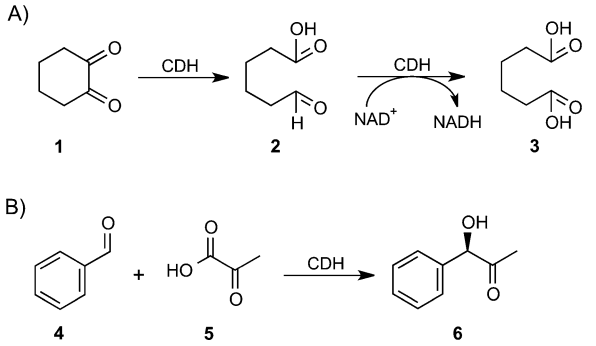
The thiamine diphosphate (ThDP)-dependent enzyme cyclohexane-1,2-dione hydrolase (CDH) was expressed in *Escherichia coli* and purified by affinity chromatography (Ni-NTA). Recombinant CDH showed the same C–C bond-cleavage and C–C bond-formation activities as the native enzyme. Furthermore, we have shown that CDH catalyzes the asymmetric cross-benzoin reaction of aromatic aldehydes and (decarboxylated) pyruvate (up to quantitative conversion, 92–99% *ee*). CDH accepts also hydroxybenzaldehydes and nitrobenzaldehydes; these previously have not (or only in rare cases) been known as substrates of other ThDP-dependent enzymes. On a semipreparative scale, sterically demanding 4-(*tert*-butyl)benzaldehyde and 2-naphthaldehyde were transformed into the corresponding 2-hydroxy ketone products in high yields. Additionally, certain benzaldehydes with electron withdrawing substituents were identified as potential inhibitors of the ligase activity of CDH.

Thiamine diphosphate (ThDP)-dependent enzymes are involved in a wide range of metabolic pathways and catalyze a broad variety of reactions. Among these are oxidative and non-oxidative decarboxylation and asymmetric C–C, C–O, C–S, and C–N bond formation, as well as C–C bond cleavage.[1] Prominent representatives include the enzymes transketolase, pyruvate dehydrogenase, pyruvate decarboxylase, acetolactate synthase, desoxyxylulose 5-phosphate synthase, benzaldehyde lyase, and benzoylformate decarboxylase (BFD).[1b]

ThDP-dependent cyclohexane-1,2-dione hydrolase (CDH, EC 3.7.1.11) is the key enzyme of an anaerobic degradation pathway of alicyclic alcohols. It catalyzes C–C bond cleavage of cyclohexane-1,2-dione (**1**) to produce 6-oxohexanoic acid (**2**) as the primary product, presumably[2] followed by oxidation of the latter to adipic acid (**3**, Scheme 1 A).[3–6] This degradation pathway of a 1,2-diketone was discovered for the denitrifying bacterium *Azoarcus* sp. strain 22Lin[7] when cultivated on cyclohexane-1,2-diol as the sole carbon source and electron donor, and nitrate as electron acceptor. Additionally, CDH is able to catalyze nonphysiological asymmetric C–C bond formation. Initially, the cross-benzoin reaction of benzaldehyde (**4**) and pyruvate (**5**) (after decarboxylation) to result in the *R*-configured 1-hydroxy-1-phenylpropan-2-one (PAC, **6**, 98% *ee*) was performed on an analytical scale (Scheme 1 B).[6]



Scheme 1. ThDP-dependent cyclohexane-1,2-dione hydrolase (CDH) catalyzes A) C–C bond-cleavage and B) asymmetric C–C bond-formation.

Until recently, CDH has been purified from its native source, *Azoarcus* sp. strain 22Lin, by a multistep chromatographic protocol. Together with the cofactors ThDP, FAD, and Mg^{II}, the enzyme monomer has a theoretical molecular mass of 64.5 kDa.[3] Whereas SDS-PAGE shows a single protein band for the monomer and size exclusion chromatography indicates that CDH is a homodimer in solution, the crystal structure shows a homotetramer with one noncovalently bound FAD and one ThDP (bound to the protein with an Mg^{II} ion that is coordinated to its diphosphate moiety) per monomer.[3–6]

The oligonucleotide and amino acid sequences of CDH do not show pronounced similarities to related enzymes, despite having typical cofactor-binding domains.[8] Interestingly, **1** is also a substrate of ThDP-dependent YerE from *Yersinia pseudotuberculosis*;[9] however, YerE does not display α-ketolase activity (observed with CDH for this substrate), but instead catalyzes the addition of activated acetaldehyde to result in a tertiary alcohol.[9] We anticipated that CDH could possess further distinctive features concerning the range of catalytic possibilities. Herein, we describe the detailed characterization of CDH-catalyzed asymmetric transformations, facilitated by heterologous production of the enzyme. The results demonstrate that CDH complements other well-known ThDP-dependent enzymes with respect to substrate range and reaction scope.

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Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/cbic.201300673.

For optimal production and isolation of CDH, a synthetic gene was designed with codon optimization for expression with a C-terminal His_6 tag from pET21a in *Escherichia coli* (see the Supporting Information). *E. coli* BL21(DE3) cells were transformed with the plasmid, grown in lysogeny broth (LB), and gene expression was induced by isopropyl-β-D-thiogalactopyranoside (IPTG). Recombinant CDH was purified by affinity chromatography (Ni-NTA). The purity of the protein was confirmed by SDS-PAGE (see the Supporting Information), and the presence of the cofactor FAD was confirmed by UV/vis spectroscopy. Approximately 15 mg of pure CDH was obtained from 1 L of culture. Although riboflavin supplement in heterologous growth cultures has been reported to optimize the expression of some FAD-dependent enzymes,[10] in the case of CDH we did not observe any effect of riboflavin (up to 50 mg L^{-1}) on the final yield of holoenzyme.

To determine the catalytic activity of recombinant CDH, both cleavage of cyclohexane-1,2-dione (**1**, Scheme 1 A) and the cross-benzoin reaction of benzaldehyde (**4**) and pyruvate (**5**, Scheme 1 B) were examined. When **1** was incubated with CDH in the absence of NAD^+, the cleavage reaction stopped at 6-oxohexanoic acid (**2**). Thus, the cleavage activity of CDH could be determined unequivocally by proton NMR spectroscopy: acidification of the aqueous enzymatic assay and extraction with $CDCl_3$ gave **2**, which displayed a well-defined signal for its aldehyde proton ($\delta_{H\text{-}6} = 9.78$ ppm, triplet, $J_{6,5} = 1.5$ Hz); furthermore, **1** seemed to exist exclusively as its 2,3-enol form ($\delta_{H\text{-}3} =$ 6.15 ppm, triplet, $J_{3,4} = 4.6$ Hz). The formation of the PAC product (**6**) was monitored by GC/MS. Preliminary experiments suggested that a 10 mM:25 mM ratio of **4** to **5** results in the highest conversion after 24 h. In the first few hours, formation of **6** showed first-order kinetics. In the linear region (0–4.7 h), the specific activity of CDH was 9.5 mU mg^{-1} purified protein (see the Supporting Information). Conversion of **4** into (*R*)-PAC ((*R*)-**6**) was 82% after 24 h, as determined by GC/MS; *ee* was 99%, as determined by chiral-phase HPLC.

Thus, we confirmed that recombinant CDH shows the same C–C bond-cleavage and C–C bond-formation activity as reported for CDH purified from its native source, *Azoarcus* sp. strain 22Lin. Furthermore, it was shown that 20–30% (*v*/*v*) DMSO had no negative effects on the conversion.[11] This allows the use of hydrophobic aromatic aldehydes in screening experiments on the carboligase activity of CDH.

The scope and limitations of the C–C bond-formation capability of CDH were determined by testing 24 monosubstituted benzaldehydes **7a**,**b**,**c**–**14a**,**b**,**c** as putative acceptors and pyruvate (**5**) as donor in the formation of the corresponding PAC derivatives **15a**,**b**,**c**–**22a**,**b**,**c** (Table 1). Overall, in terms of conversion and *ee* of the PAC products, benzaldehydes with one electron-rich substituent (OH, OMe, Me) in the *ortho*, *meta*, or *para* position (substrates **11**–**13**) gave slightly better results than **7**–**10** and **14** (electron-withdrawing groups; F, Cl, Br, I, NO_2). Benzaldehydes substituted in the *para* or *meta* positions showed better conversion than the corresponding *ortho*-substituted derivatives.

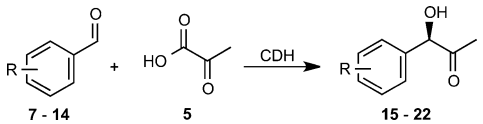
Notably, CDH accepted hydroxy- and nitrobenzaldehydes **11** and **14**, which are not (or only in rare cases)[12] known as substrates of other ThDP-dependent enzymes. There was no conversion with *o*-nitrobenzaldehyde (**14a**), which was thus the only tested aromatic aldehyde not to be accepted by CDH. Unexpectedly, CDH showed particularly low conversion of 2-fluorobenzaldehyde (**7a**; see below).

All 18 PAC products with determined *ee* values (Table 1) were assigned as *R*-configured, as judged by the appearance of a negative band centered at 270–290 nm in circular dichroism spectra.[13,14] All 18 products were highly enantioenriched, with *ee* values in the range of 92–99%.

As *para*-substituted and electron-rich benzaldehydes were accepted best in terms of conversion, we applied sterically more demanding aromatic aldehydes **23**–**29** in the cross-benzoin reaction with pyruvate (Scheme 2).

In general, these substrates were almost completely converted, and products **30**–**35** were obtained virtually enantiopure. The reaction of 4-(*tert*-butyl)benzaldehyde (**26**→**33**, 98% conversion) is noteworthy. Although the 2-hydroxy ketone **33** was obtained with a relatively lower *ee* (92%), it should be emphasized that compounds bearing *tert*-butyl groups are notoriously poor substrates in enzymatic transformations.[15] This is because of the high hydrophobicity of *tert*-butylated substrates (thus low solubility in aqueous media) and the steric hindrance imposed by the *tert*-butyl group. In our example, DMSO (20% *v*/*v*) as the solubility promoter undoubtedly alleviated the solubility challenge.

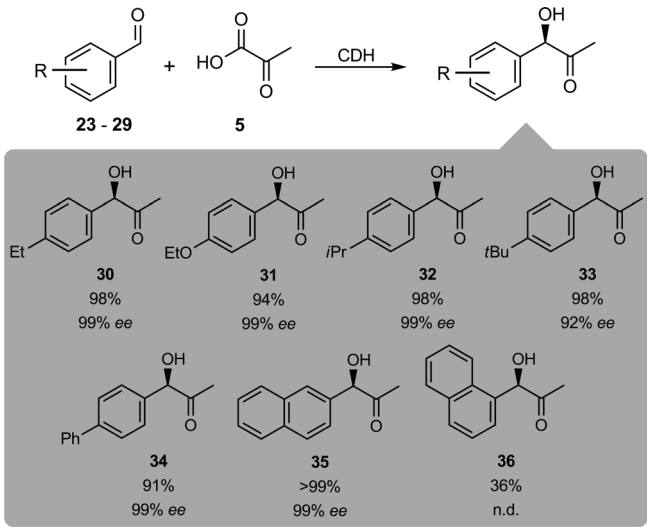
Table 1. CDH-catalyzed conversion (%) of **7**–**14** and enantiomeric excess (% *ee* in brackets) for the formation of PAC derivatives **15**–**22**.[a]



7 - 14 5 15 - 22

R	F	Cl	Br	I	OH	OMe	Me	NO_2
substrate	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**
product	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**
ortho (**a**)	5 (n.d.)	6 (n.d.)	24 (99)	45 (96)	19 (n.d.)	37 (99)	62 (99)	0 (–)
meta (**b**)	47 (98)	34 (98)	49 (96)	51 (94)	82 (99)	96 (92)	91 (98)	20 (n.d.)
para (**c**)	68 (96)	82 (96)	69 (95)	81 (96)	30 (98)	85 (98)	97 (96)	5 (n.d.)

[a] Conditions: pyruvate (25 mM), aromatic aldehyde (10 mM), CDH (1 mg mL^{-1}), buffer A (50 mM MES, 1 mM $MgSO_4$, 0.5 mM ThDP, pH 6.5), DMSO (20–30%, *v*/*v*), 30 °C, 48 h; % conversion was determined by GC/MS. Enantiomeric excess was determined by chiral-phase HPLC; n.d. = not determined.



Scheme 2. Screening sterically demanding aromatic aldehydes as acceptors with pyruvate (**5**, donor) in the CDH-catalyzed cross-benzoin reaction. Conditions as in Table 1.

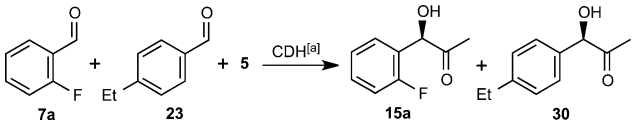
Moreover, CDH used naphthaldehydes efficiently as acceptor substrates, although the sterically hindered 1-naphthaldehyde (**29**→**36**) showed only 36% conversion (>99% conversion of the less hindered 2-naphthaldehyde (**28**→**35**)). Previously, 2-naphthaldehyde (**28**) has been applied as a donor substrate in BFD-catalyzed cross-benzoin condensation: in the presence of solubilizing agents, only 34% conversion was obtained after 72 h.[16] In addition, 1-naphthaldehyde (**29**) was not accepted at all by BFD, thus further highlighting the significance of our results with CDH. The transformations of 4-(*tert*-butyl)benzaldehyde (**26**→**33**) and 2-naphthaldehyde (**28**→**35**) were also performed on a semipreparative scale. The 2-hydroxy ketone products **33** and **35** were obtained in 90% (28 mg) and 87% (26 mg) isolated yields, respectively.

In contrast to the broad acceptor substrate spectrum, the donor substrate range of the CDH-catalyzed cross-benzoin reaction with benzaldehyde (**4**) was, in effect, limited to pyruvate (82% conversion). Aliphatic α-oxo acids larger than 2-oxobutanoic acid (1.3% conversion) were not accepted at all, and neither were 2-oxosuccinate, 2-oxoglutarate, or hydroxypyruvate (the last substrate was tested with 4-ethylbenzaldehyde as acceptor). In the absence of an aromatic acceptor aldehyde, highly enantioenriched (*S*)-acetoin was formed by homocoupling of pyruvate.[17]

Analysis of the data in Table 1 with respect to the conversion of *ortho*-substituted halobenzaldehydes reveals a trend that is contrary to what was expected. On the basis of the steric requirements and the electrophilicity of the halo substituents, 2-fluorobenzaldehyde (**7a**) should be accepted best as a substrate, and 2-iodobenzaldehyde (**10a**) should be accepted least. (This trend is regularly observed with other ThDP-dependent enzymes.)[9,18] However, CDH catalysis displayed the opposite outcome (Table 1). Accordingly, we hypothesized that 2-fluorobenzaldehyde (**7a**) might act as an inhibitor of the carboligase activity of CDH. To test this, we set up a competition experiment with **7a** (a potential inhibitor), 4-ethylbenzaldehyde (**23**, one of the best acceptors), and pyruvate (**5**, donor).

In the absence of **7a**, conversion of **23** to 1-(4-ethylphenyl)-1-hydroxypropan-2-one (**30**) was 95% after 24 h (Table 2, entry 1) and 98% after 48 h (entry 2). In the presence of equimolar amounts of **7a** and **23** the conversion was just 1% (entry 3); interestingly, 1-(2-fluorophenyl)-1-hydroxypropan-2-one (**15a**) was detected in only trace amounts. Lowering the **7a**:**23** ratio revealed that **7a** has a strong negative impact on the formation of 1-(4-ethylphenyl)-1-hydroxypropan-2-one (**30**): 34% conversion of **23**→**30** when present as low as 10% relative to **23** (entry 5).

Table 2. Competition experiments with 2-fluorobenzaldehyde (**7a**), 4-ethylbenzaldehyde (**23**), and pyruvate (**5**).[a]



	Quantity [mM]				Product [%]
	7a	**23**	**5**	**15a**	**30**
1	–	10	25	–	95
2[b]	–	10	25	–	98
3	10	10	25	2	1
4	5	10	25	6	1
5	1	10	25	61	34
6	0.1	10	25	n.d.[c]	82

[a] Conditions: pyruvate (25 mM), benzaldehydes **7a** and **23** (see Table 2), CDH ($c = 1$ mg mL^{-1}), buffer A (see Table 1), DMSO (20–30% *v*/*v*), 30 °C, 24 h; % conversion (each value is an average from two independent enzymatic assays) was determined by GC/MS. [b] After 48 h. [c] Not detected.

We then turned our attention to other aromatic aldehydes possessing (highly) electron-withdrawing substituents. A potential inhibitory effect on the formation of **30** was tested in independent competition experiments under conditions analogous to those listed in Table 2, entry 4. As anticipated, 2-chlorobenzaldehyde (**8a**), as well as 2,6-difluoro-, 2,4,5-trifluoro-, and pentafluorobenzaldehyde, showed the same effect as **7a**: each of these benzaldehydes yielded ≤6% conversion into the corresponding PAC products when incubated with pyruvate (**5**), and inhibited the formation of **30** in the same manner as **7a** (<2% conversion for **23**→**30**). Detailed kinetic studies are required to obtain a mechanistic explanation of these results and to confirm the role of these electron-deficient benzaldehydes as potential inhibitors of the ligase activity of CDH.

In summary, we have described the heterologous expression in *E. coli* and purification of recombinant His-tagged cyclohexane-1,2-dione hydrolase (CDH). In addition to its physiological C–C bond-cleavage activity, CDH catalyzes the asymmetric cross-benzoin reaction of a broad variety of aromatic aldehydes and pyruvate (up to quantitative conversion, 92–99% *ee*). In the case of the sterically demanding 4-(*tert*-butyl)benzaldehyde (**26**) and 2-naphthaldehyde (**28**), the respective 2-hydroxy ketone products (**33** and **35**) were obtained in high yield. Notably, CDH accepts several benzaldehydes, such as hy-

droxy- and nitrobenzaldehydes; these are not (or only in rare cases) known as substrates of other ThDP-dependent enzymes. An inhibitory effect of benzaldehydes with electron-withdrawing substituents on the carboligase activity was observed.

Hence, CDH expands the range of well-known ThDP-dependent enzymes suitable as catalysts in asymmetric carboligation reactions. The straightforward production and stability of this enzyme, as well as its unique behavior as a powerful multifunctional catalyst, make it an interesting starting point for synthetic applications and mechanistic studies.

Experimental Section

Cloning: The synthetic, codon-optimized, *cdh* gene from *Azoarcus* sp. 22Lin (GeneArt, Life Technologies) was cloned into a pET21a vector (Novagen), between NdeI and XhoI restriction sites. Omission of the gene's stop codon allowed expression with the plasmid-encoded C-terminal His_6 tag.

Expression and purification of CDH: For the production of His-tagged CDH, *E. coli* BL21(DE3) was transformed with pET21a carrying the *cdh* gene. A preculture was grown in LB (7 mL) with ampicillin (100 $\mu g\,mL^{-1}$) at 37 °C for 24 h with shaking (140 rpm). From this, an aliquot was incubated (25 °C, 130 rpm) in fresh LB (500 mL) with ampicillin (100 $\mu g\,mL^{-1}$). Upon reaching $OD_{600} = 0.6–0.7$ (ca. 5.5 h), IPTG (0.02 mM) was added to induce gene expression. After 17–24 h at 25 °C (130 rpm), cells were harvested by centrifugation (5200 *g*, 40 min, 4 °C). From the 500 mL culture, 2.5–3.0 g of cells were obtained. The cell pellet was resuspended in buffer A (15 mL; MES (2-(*N*-morpholino)ethanesulfonic acid, 50 mM, pH 6.5), $MgSO_4$ (1 mM), ThDP (0.5 mM)) and lysed by a French press. Cell debris was removed by centrifugation (6000 *g*, 45 min, 4 °C), and the lysate (20 mL) was incubated with Ni-NTA resin (2 mL) at 0 °C for 1 h. After washing with buffer A containing imidazole (20 then 50 mM), recombinant CDH was eluted in buffer A containing imidazole (300 mM). The combined elution fractions were desalted by gel-permeation chromatography in buffer A in a Sephadex column (GE Healthcare). The purity of the protein was confirmed by SDS-PAGE (see the Supporting Information). Approximately 15 mg purified protein was obtained from 1 L cell culture.

CDH-catalyzed asymmetric benzoin reaction of benzaldehydes and pyruvate (analytical scale): Enzymatic reactions on an analytical scale were performed in buffer A (1.5 mL) containing DMSO (20–30% *v/v*). The aromatic aldehyde (final concentration 10 mM) was applied as a DMSO solution; sodium pyruvate (25 mM) and the enzyme (1 $mg\,mL^{-1}$) were added. All reactions were conducted at 30 °C and 300 rpm in a Thermomixer (Eppendorf). After 48 h, the enantiomeric excess was determined by chiral-phase HPLC after extraction of an aliquot (150 μL) of the enzyme assay with ethyl acetate (1×200 μL). Conversion was determined by GC/MS after extraction of another aliquot (150 μL) with ethyl acetate (1× 150 μL). For analytical details of the PAC products, see the Supporting Information.

CDH-catalyzed synthesis of 33 and 35 (semipreparative scale): A solution of 4-(*tert*-butyl)benzaldehyde (**26**, 25.1 μL, 24.3 mg, 0.150 mmol, final concentration in the reaction assay 10 mM) in DMSO (3.75 mL, final concentration 25%, *v/v*) or 2-naphthaldehyde (**28**, 23.4 mg, 0.150 mmol, final concentration 10 mM) in DMSO (4.50 mL, final concentration 30%, *v/v*) was added to a solution of sodium pyruvate (41.6 mg, 0.378 mmol, final concentration 25 mM) in buffer A (final total volume 15 mL) in a 15 mL Falcon tube. CDH (1 $mg\,mL^{-1}$) was added, and the mixture was incubated at 30 °C with shaking (400 rpm) for 24 h (for **28**) or 48 h (for **26**) until GC/MS indicated 99% conversion. The reaction mixture was extracted with MTBE (3×15 mL). The combined organic layers were dried over $MgSO_4$, and the volatiles were removed under reduced pressure. The crude product was isolated with an Isolera preparative flash purification system (Biotage AB, Uppsala, Sweden).

(*R*)-1-(4-*tert*-Butylphenyl)-1-hydroxypropan-2-one (**33**) was obtained in 90% yield (28 mg). (*R*)-1-Hydroxy-1-(naphthalen-2-yl)propan-2-one (**35**) was obtained in 87% yield (26 mg).

Acknowledgements

This work has been supported by the German Research Foundation (DFG) through grants SPP 1319 (to P.M.H.K), FOR 1296 (to M.M.), and AN 676/1 (to S.L.A.A.).

Keywords: asymmetric catalysis · C–C bond-formation · carboligation · enzyme catalysis · ThDP

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Received: October 22, 2013
Published online on January 16, 2014