Biocatalysis

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A Tailor-Made Chimeric Thiamine Diphosphate Dependent Enzyme for the Direct Asymmetric Synthesis of (*S*)-Benzoins**

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In memory of Ayhan S. Demir

Abstract: *Thiamine diphosphate dependent enzymes are well known for catalyzing the asymmetric synthesis of chiral α-hydroxy ketones from simple prochiral substrates. The steric and chemical properties of the enzyme active site define the product spectrum. Enzymes catalyzing the carboligation of aromatic aldehydes to (S)-benzoins have not so far been identified. We were able to close this gap by constructing a chimeric enzyme, which catalyzes the synthesis of various (S)-benzoins with excellent enantiomeric excess (> 99 %) and very good conversion.*

Thiamine diphosphate (ThDP) dependent enzymes are proven catalysts for the stereo- and regioselective synthesis of chiral α-hydroxy ketones through asymmetric C–C bond formation starting from aldehydes, α-keto acids, and ketones.[1] Whereas enzymes are available for the enantiocomplementary synthesis of a broad range of symmetrical and mixed aliphatic, araliphatic, and aromatic products,[2] so far no biocatalyst is available to selectively access (*S*)-benzoins starting from benzaldehydes. Instead, other enzymatic routes became available, which involve kinetic resolution as well as deracemization of *rac*-benzoins,[3] or asymmetric reduction of 1,2-diarylethane-1,2-diones (benzils).[4] Although these approaches are mostly characterized by high stereoselectivity, they all share the same drawback: the racemic or prochiral starting material must be chemically synthesized beforehand. Such additional steps reduce the eco-efficiency and sustainability of the process compared to the direct enzymatic synthesis of (*S*)-benzoins starting from commercially available benzaldehydes. This direct synthesis is still a unique feature of nonenzymatic synthetic organic chemistry.[5]

Whereas the *R*-selective enzymatic synthesis of benzoins has already been achieved,[6] *S*-selective synthesis is limited by the specific active-site architecture of ThDP-dependent enzymes (Figure 1). Introduction of the *S*-pocket concept enabled the design of an *S*-selective variant of pyruvate decarboxylase from *Acetobacter pasteurianus* (*Ap*PDC) for the formation of (*S*)-phenylacetylcarbinol (PAC).[7] Replacement of a glutamate residue (E469) by glycine allowed benzaldehyde as an acceptor to bind predominantly in antiparallel orientation relative to the ThDP-bound donor, which is a prerequisite for *S*-selectivity. However, *Ap*PDC-E469G only provides a small donor-binding site that preferentially stabilizes small aliphatic donor substrates, thereby preventing the formation of (*S*)-benzoin [(*S*)-**1a**] (Figure 1 A). Benzaldehyde lyase from *Pseudomonas fluorescens* (*Pf*BAL) is a powerful but strictly *R*-selective catalyst for the

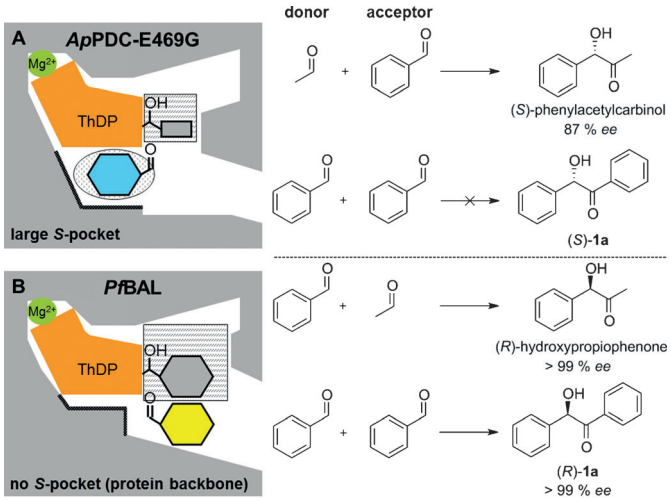

Figure 1. A schematic presentation of the scope and limitations of carboligations catalyzed by *Ap*PDC-E469G and *Pf*BAL. (*S*)-Benzoin (**1a**) formation is prevented either by a small donor-binding site (A), or an *S*-pocket that is inaccessible for the antiparallel orientation of the acceptor benzaldehyde relative to the ThDP-bound donor benzaldehyde (as a hydroxybenzyl group; B). Dashed rectangle: donor-binding site; dotted circle: *S*-pocket region; rectangle: acetaldehyde; hexagon: benzaldehyde; gray: donor; cyan: antiparallel-oriented acceptor, which leads to the respective (*S*)-product; yellow: parallel-oriented acceptor, which leads to the respective (*R*)-product.

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synthesis of **1a**.[6b] In contrast to *Ap*PDC, *Pf*BAL provides a large donor-binding site, which is ideal for stabilizing benzaldehyde in that position. However, owing to a missing *S*-pocket, *Pf*BAL only allows the parallel arrangement of the donor and acceptor benzaldehydes prior to carboligation. Furthermore, *S*-pocket engineering is limited in *Pf*BAL by the position of the protein backbone of the respective α-helix in the *S*-pocket region (Figure 1B).

To solve the long-standing problem of enzymatic (*S*)-benzoin synthesis, a rational hybridization approach was followed, in which the active-site characteristics of the variant *Ap*PDC-E469G[7] and *Pf*BAL were combined. Two approaches were conceivable: 1) the introduction of a large *S*-pocket into *Pf*BAL, or 2) the extension of the donor-binding site of *Ap*PDC-E469G. Herein, we show that the second approach indeed resulted in the first tailor-made *S*-selective ThDP-dependent enzyme variant for the formation of various benzoins.

A combination of modeling studies and a comprehensive sequence analysis of the amino acid distribution in 186 homologous PDC sequences and 43 BAL sequences led to the identification of a threonine residue (T384) as the pivotal factor that influences the size of the donor-binding site in *Ap*PDC-E469G. T384 limits the space for benzaldehyde in the donor-binding site and is conserved in 92% of *Ap*PDC homologous sequences (Figure 2A), whereas glycine was found at the equivalent position in all *Pf*BAL homologous sequences. T384 in *Ap*PDC-E469G was replaced with glycine through site-directed mutagenesis in order to mimic the *Pf*BAL donor-binding site. As a result, an enlarged donor-binding site with putatively sufficient space for benzaldehyde was obtained (Figure 2B). Moreover, short molecular dynamics simulations revealed that a neighboring tryptophan residue (W388) underwent minor conformational changes, probably because of interactions with the ThDP-bound benzaldehyde donor (hydroxybenzyl), thereby further opening up the donor-binding site.

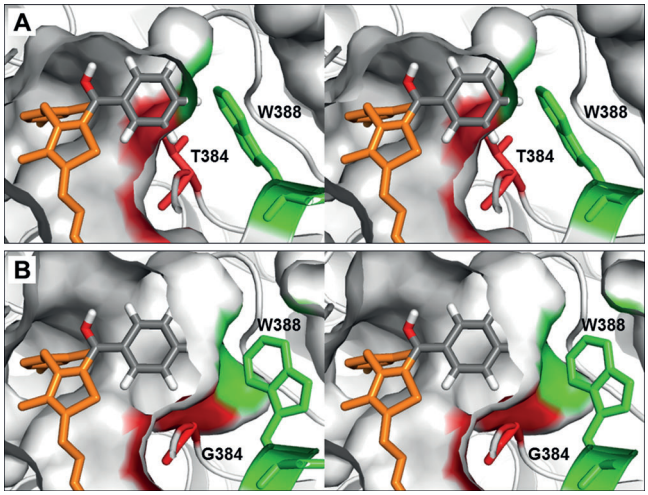

Figure 2. Stereoview of the donor-binding sites of *Ap*PDC-E469G (A) and *Ap*PDC-E469G/T384G (B) with benzaldehyde bound as a hydroxybenzyl group (gray) to C2 of ThDP (orange). A) *Ap*PDC-E469G is not able to properly bind benzaldehyde in the small donor-binding site, which is mainly restricted by T384 (red). B) The donor-binding site could be opened for benzaldehyde by replacing T384 with glycine. Moreover, the equilibrated structure revealed conformational changes to W388 (green) that additionally opened the donor-binding site.

Biochemical characterization of the new variant proved that the single mutation of T384 to glycine indeed altered the chemoselectivity of *Ap*PDC-E469G, which subsequently preferred benzaldehyde as the donor. Whereas no significant formation of **1a** in the homocoupling of benzaldehyde was observed with *Ap*PDC-E469G (Table 1, entry 1), double

Table 1: Enzymatic synthesis of (*S*)-**1a** as catalyzed by *Ap*PDC variants.[a]

Entry	*Ap*PDC variant	*ee* [%][b]	Conv. [%][c]
1	E469G	n.d.[d]	< 1
2	E469G/T384G	59	52
3	E469G/T384G/I468G	66	23
4	E469G/T384G/I468A	87	95
5	E469G/T384G/I468V	76	40
6	E469G/T384G/I468V/W543F	95	36

[a] Reaction conditions: 50 mM triethanolamine buffer, pH 8.0, 2 mM $MgSO_4$, and 0.1 mM ThDP; 1 mg mL^{-1} enzyme; 18 mM benzaldehyde; 20°C, 6 h; [b] determined by chiral-phase HPLC; [c] Determined by chiral-phase HPLC based on the consumption of benzaldehyde; [d] Not determined.

variant *Ap*PDC-E469G/T384G catalyzed the benzoin formation with 52% conversion under the tested conditions (entry 2). The preference for benzaldehyde as the donor was also demonstrated in the mixed carboligation of acetaldehyde and benzaldehyde by variant *Ap*PDC-E469G/T384G, which resulted in the formation of a mixture of **1a** and 2-hydroxypropiophenone (2-HPP), whereas PAC, the main product obtained with wild-type *Ap*PDC or variant *Ap*PDC-E469G,[7] was only detected in trace amounts. Furthermore, variant *Ap*PDC-E469G/T384G catalyzed both the synthesis of (*S*)-**1a** from benzaldehyde with moderate stereoselectivity (59% *ee*, entry 2) and mixed carboligation towards 2-HPP with good *S*-selectivity (91% *ee*).

To improve the moderate *S*-selectivity of *Ap*PDC-E469G/T384G, two strategies are possible: stabilization of the antiparallel ("*S*-pathway") or destabilization of the parallel acceptor orientation ("*R*-pathway") prior to carboligation.[8] To suppress the *R*-pathway in *Ap*PDC-E469G/T384G, the active site was examined for residues that potentially stabilize the acceptor benzaldehyde in the parallel orientation. By performing molecular modeling, I468 and W543 were identified (Figure 3A) as residues that could stabilize parallel-oriented benzaldehyde through nonpolar interactions or π-stacking.

I468 was replaced with valine, glycine, or alanine to increase the distance of the respective side chain to parallel-oriented benzaldehyde and thus disrupt the stabilization of the *R*-pathway (Figure 3B). In all cases, the new variants showed improved *S*-selectivity for the formation of (*S*)-**1a** (Table 1, entries 3–5). Out of the different variants, the triple variant E469G/T384G/I468A performed best with respect to *ee* (87%, *S*) and conversion (95%) under standard reaction conditions. The high conversion is particularly surprising

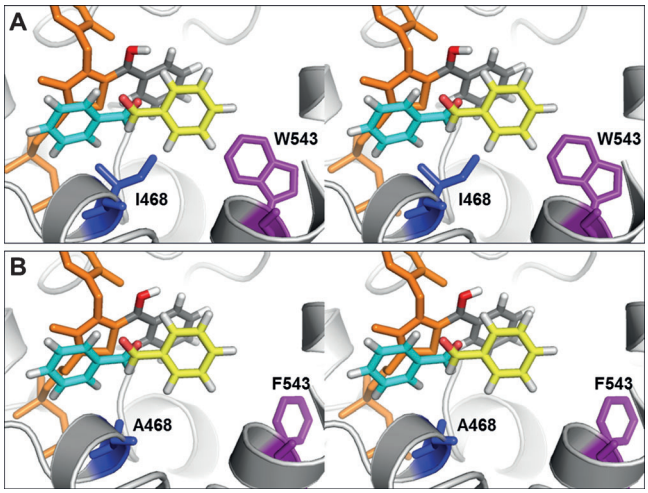

Figure 3. Stereoview representation of the possible stabilization of the parallel-oriented acceptor benzaldehyde (yellow) in the active sites of *Ap*PDC-E469G/T384G (A) and *Ap*PDC-E469G/T384G/I468A/W543F (B). A) In E469G/T384G, parallel-oriented benzaldehyde is putatively stabilized by I468 (blue) and W543 (purple), neither of which directly influence its antiparallel orientation (cyan). B) In variant E469G/T384G/I468A/W543F, the stabilization of parallel-oriented benzaldehyde by A468 (blue) and F543 (purple) is no longer possible.

because multiple active-site mutations frequently result in a drastic decrease in enzymatic activity, as has been demonstrated for other ThDP-dependent enzymes.[8]

To further improve the stereoselectivity of variant E469G/T384G/I468A, a fourth mutation at the position of tryptophan 543 was introduced. W543 is part of the C-terminal α-helix, which covers the entrance to the active site of *Ap*PDC (Figure 3A), and of an aromatic cluster (for details, see Chapter 2 of the Supporting Information) that might be relevant for structural stabilization. This position was subjected to site-saturation mutagenesis by using NDT codon degeneracy. Only few variants showed significant carboligation activity during screening, but all of the active variants were *S*-selective for the formation of **1a**. Among these variants, E469G/T384G/I468A/W543F revealed improved *S*-selectivity for the synthesis of **1a**, with 95% *ee* and a conversion of 36% (Table 1, entry 6). In comparison to W543, the increased distance between the phenyl ring of the parallel-oriented acceptor benzaldehyde and F543 (6.8 Å compared to 3.7 Å) is assumed to prevent stabilizing interactions (Figure 3B). Furthermore, the physicochemical properties at position 543 were preserved by the substitution of tryptophan by phenylalanine, which might be beneficial to maintaining the structural stability conferred by the aromatic cluster in the *Ap*PDC variant. In addition to improved *S*-selectivity in the homocoupling reaction of benzaldehyde, *Ap*PDC-E469G/T384G/I468A/W543F also revealed enhanced stereoselectivity for the synthesis of (*S*)-2-HPP (>99% *ee*) starting from benzaldehyde as the donor and acetaldehyde as the acceptor substrate.

To evaluate the synthetic potential of the newly designed variants, a substrate screening with substituted benzaldehyde derivatives was performed with *Ap*PDC-E469G/T384G/I468A and *Ap*PDC-E469G/T384G/I468A/W543F (Table 2) under optimized reaction conditions (reduced temperature of 15°C) for the synthesis of (*S*)-**1a**. *meta*-Substituted benzaldehyde derivatives turned out to be the best substrates in the homocoupling reaction in terms of *S*-selectivity and conversion. Except for 3-fluorobenzaldehyde (**1e**), all of the *meta*-substituted benzaldehydes (Table 2, entries 5–9) were transformed with higher *S*-selectivity than benzaldehyde (Table 2, entry 1). The reactions with variant E469G/T384G/I468A/W543F again showed higher *S*-selectivity and lower conversion than those with variant E469G/T384G/I468A. Remarkably, variant E469G/T384G/I468A/W543F catalyzed the synthesis of enantiopure **1f–i** (>99% *ee* (*S*), Table 2, entries 6–9). These synthesis reactions could be successfully scaled-up from analytical scale (300 µL) to preparative scale (20 mL) with final product concentrations of up to 4 g L^{-1} (yields of isolated product 61–85%, Table 2).

Table 2: Synthesis of benzoins (**1a–l**) as catalyzed by *Ap*PDC variants.[a]

Entry	Ar	Product	E469G/T384G/I468A		E469G/T384G/I468A/W543F	
			ee [%][b]	Conv. [%][c] (yield [%])[d]	*ee* [%][b]	Conv. [%][c] (yield [%])[d]
1	C_6H_5	**1a**	89 (*S*)	92 (85)	98 (*S*)	26 (66)
2	2-FC_6H_4	**1b**	58 (*R*)	36	21 (*R*)	<5
3	2-ClC_6H_4	**1c**	75 (*R*)	<5	n.d.	n.c.
4	2-$MeOC_6H_4$	**1d**	n.d.[e]	n.c.	n.d.	n.c.
5	3-FC_6H_4	**1e**	87 (*S*)	80 (82)	93 (*S*)	36
6	3-ClC_6H_4	**1f**	91 (*S*)	97	>99 (*S*)	48 (72)
7	3-$BrOC_6H_4$	**1g**	95 (*S*)	85 (84)	>99 (*S*)	30
8	3-IC_6H_4	**1h**	96 (*S*)	30 (70)	>99 (*S*)	11
9	3-$MeOC_6H_4$	**1i**	98 (*S*)	93	>99 (*S*)	58 (61)
10	4-FC_6H_4	**1j**	64 (*S*)	53	85 (*S*)	<5
11	4-ClC_6H_4	**1k**	>99 (*R*)	10	n.d.	n.c.
12	4-$MeOC_6H_4$	**1l**	n.d.	<5	n.d.	n.c.

[a] Reaction conditions: see Table 1, reaction temperature: 15°C; [b, c] See Table 1; [d] Yields of isolated product after preparative synthesis and conversion of >90% after 24 h and 48 h, (reaction conditions: see Chapter 4 of the Supporting Information); [e] Not determined; [f] No conversion.

In contrast to *meta*-substituted benzaldehydes, *ortho*- and *para*-substituted benzaldehydes, with the exception of 4-fluorobenzaldehyde (**1j**), were converted with low conversion to yield an excess of the *R*-enantiomer.

These results confirm our findings from recent studies on 2-succinyl-5-enolpyruvyl-6-hydroxy-3-cyclohexene-1-carboxylate synthase (MenD), which revealed that *meta*-substituted benzaldehydes are converted with higher *S*-selectivity and conversion than *ortho*- and *para*-substituted benzaldehydes and unsubstituted benzaldehyde.[8,9] However, the reasons for the preference for *meta*-substituted benzaldehydes in *S*-

selective carboligation reactions and the switch in stereoselectivity in the case of *ortho*- and *para*-substituted benzaldehydes are still not understood. In this case, a combination of steric and electronic interactions between the substrate and the *S*-pocket might influence the stereoselectivity. The elucidation of this phenomenon is now the subject of further investigation.

In summary, the rational design of a hybrid substrate-binding site, which combines the active-site characteristics of the *S*-selective *Ap*PDC-E469G variant and *Pf*BAL, enabled the creation of a new biocatalyst for the synthesis of different (*S*)-benzoins with excellent *ee* values and good conversion when starting from commercially available benzaldehydes. These results highlight the robustness of ThDP-dependent enzymes with respect to active-site mutations, as well as their tremendous catalytic potential in asymmetric carboligation reactions. Moreover, this hybridization approach might open the field for the combinatorial assembly of selectivity-determining modules from different ThDP-dependent enzymes and offer a new perspective on thiamine catalysis with respect to the design of variants with novel catalytic activities.

Experimental Section

All chemicals were purchased from Sigma Aldrich. Benzaldehydes were freshly distilled before use. The generation, expression, and purification of *Ap*PDC variants are described in the Supporting Information. Descriptions of sequence and structural analyses, as well as reaction details and product analytics, can also be found in the Supporting Information.

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