Alteration of the Substrate Specificity of Benzoylformate Decarboxylase from *Pseudomonas putida* by Directed Evolution

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Alteration of the substrate specificity of thiamin diphosphate (ThDP)-dependent benzoylformate decarboxylase (BFD) by error-prone PCR is described. Two mutant enzymes, L476Q and M365L-L461S, were identified that accept ortho*-substituted benzaldehyde derivatives as donor substrates, which leads to the formation of 2-hydroxy ketones. Both variants, L476Q and M365L-L461S, selectively catalyze the formation of enantiopure (S)-2-hydroxy-1-(2-methylphenyl)propan-1-one with excellent yields, a reaction which is only poorly catalyzed by the wild-type enzyme. Different* ortho*-substituted benzaldehyde derivatives, such as 2-chloro-, 2-methoxy-, or 2-bromobenzaldehyde are accepted as donor substrates by both BFD variants as well and conversion with acetaldehyde resulted in the corresponding (S)-2-hydroxy-1-phenylpropan-1-one derivatives. As deduced from modeling studies based on the 3D structure of wild-type BFD, reduction of the side chain size at position L461 probably results in an enlarged substrate binding site and facilitates the initial binding of* ortho*-substituted benzaldehyde derivatives to the cofactor ThDP.*

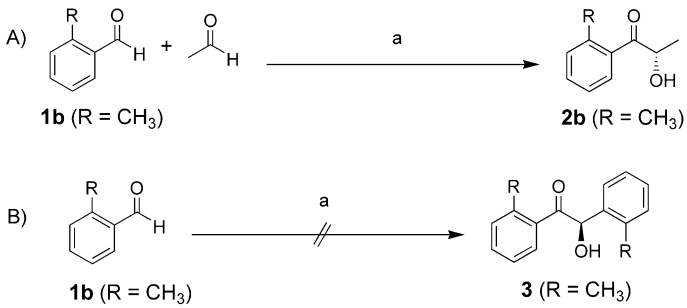
KEYWORDS:

asymmetric synthesis · directed evolution · enzyme catalysis · hydroxy ketones · protein engineering

Introduction

Biocatalysis is an important option when facing the challenge of producing single enantiomers.[1–3] However, as a result of their natural function, many enzymes have a narrow substrate specificity. Protein engineering is a promising approach to generate proteins with new or improved properties. These modified biocatalysts can be obtained by using rational or random strategies. The latter approaches have the advantage that a detailed knowledge about the relationship between molecular structure and function is not required. Various methods have been developed to randomly mutagenize a gene encoding an enzyme.[4] Every protein can be evolved without any knowledge of its structure by performing a functional screening or selection.[5] It has been demonstrated that directed evolution can lead to enzymes with thermal stability,[6, 7] organic solvent resistance,[8–10] inverted enantioselectivity,[11–13] and new substrate specificity.[14–16]

Thiamin diphosphate (ThDP)-dependent benzoylformate decarboxylase (BFD, E.C. 4.1.1.7) from *Pseudomonas putida* is an efficient catalyst for the enantioselective formation of 2-hydroxy ketones.[17, 18] Like other members of the family of ThDP-dependent decarboxylases,[19] BFD is able to catalyze this acyloin-type condensation as a side reaction, while the main reaction is the nonoxidative decarboxylation of benzoylformate. BFD has been used as a catalyst for the enantioselective formation of (*S*)-2-hydroxy-1-phenylpropan-1-one ((*S*)-2-HPP, **2a**, structure as for **2b** with R = H, see Scheme 1) from benzaldehyde and acetaldehyde, as well as for the formation of (*R*)-benzoin from two molecules of benzaldehyde.[17, 20]



Scheme 1. *A) Product of the carboligation of* ortho*-methylbenzaldehyde and acetaldehyde mediated by the BFD variants; B) carboligation of two molecules of* ortho*-methylbenzaldehyde to form (R)-2,2′-dimethylbenzoin cannot be performed by using the BFD variants L476Q or M365L-L461S. a) BFD variant, KPi buffer (pH 7.0), Mg^{2+}, ThDP, 20% DMSO, 30 °C. KPi, potassium phosphate; DMSO, dimethylsulfoxide.*

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Advantageously, aldehydes can be used without previous decarboxylation of the corresponding 2-keto acids.[21] We have previously reported that BFD catalyzes the ligation of a broad range of different aromatic, heteroaromatic, and even cyclic aliphatic and conjugated olefinic aldehydes as acyl donors to acetaldehyde, which acts as the acyl acceptor.[17, 18, 22] The best results with respect to the enantiomeric excess (*ee*) of the resulting 2-hydroxy ketones were obtained with *meta*- and *para*-substituted benzaldehydes. *ortho*-Substituted benzaldehyde derivatives, except 2-fluorobenzaldehyde, are only poorly accepted as donor substrates by the wild-type enzyme, probably because of steric hindrance.

In contrast to BFD, benzaldehyde lyase (BAL, E.C. 4.1.2.38) from *Ps. fluorescens*, another ThDP-dependent enzyme, accepts aromatic aldehydes substituted in the *ortho*-posititon and reaction leads to the formation of (*R*)-*o*-2-HPP analogues when these aldehydes are converted with acetaldehyde.[23] By using these two enantiocomplementary enzymes most 2-HPP analogues except *ortho*-substitued (*S*)-2-HPP derivatives can be synthesized in either enantiomeric form.

While BFD variants that enable access to *ortho*-di-substituted benzoins could not be identified, we found two variants that accept *ortho*-substituted benzaldehyde derivatives as acyl donors, which results in the enantioselective formation of *ortho*-substituted (*S*)-2-HPP derivatives in good yields on an analytical and a preparative scale.

Results

Screening of a BFD random mutagenesis library

We used the polymerase chain reaction (PCR) to perform an error-prone mutagenesis based on the wild-type BFD gene with a C-terminal hexahistidine tail. In order to identify carboligase-active clones, 4×10^3 clones were screened with the substrates benzaldehyde and acetaldehyde by using a colorimetric assay, which is based on the potential of the formed 2-hydroxy ketones to reduce 2,3,5-triphenyltetrazolium chloride to the intense-red-colored formazane.[8, 24] 50% of the clones tested showed carboligation activity in the range of that of the wild-type enzyme. Sequencing of ten randomly picked clones of the library revealed 1 to 3 random point mutations per 1.6 kb gene. Those clones that showed carboligation activity were rescreened with *ortho*-methyl-benzaldehyde as a substrate. Carboligation of this aldehyde should lead to the *ortho*-di-substituted benzoin **3** (Scheme 1 B), a reaction which is not performed by the wild-type enzyme.

Screening of the BFD random mutagenesis library resulted in the identification of two clones that induce the development of the red color indicating the presence of a 2-hydroxy ketone. Sequencing of the mutated BFD genes showed that one mutant contained one base substitution leading to the amino acid exchange L476Q, whilst the other mutant contained three base substitutions resulting in two amino acid exchanges M365L-L461S and one silent mutation (CTT→CTC, L511). Both mutants were expressed in *Escherichia coli* SG 13009 and the enzymes were purified by metal chelate affinity chromatography.

Characterization of the BFD variants L476Q and M365L-L461S

Remarkably, the variant L476Q had also been identified in a screening of a BFD random library for improved carboligation activity in the presence of water-miscible organic solvents[8] and was characterized in detail. With acetaldehyde and benzaldehyde as substrates this variant showed a fourfold higher carboligation activity in the absence of DMSO and a fivefold higher carboligation activity in the presence of DMSO, compared to the wild-type enzyme (Table 1). The variant M365L-L461S retained only 9% of the wild-type BFD carboligase activity.

Suprisingly, the mutations positively effect the enantioselectivity with respect to the product (*S*)-2-HPP (**2 a**), as both catalysts are more stereoselective than the wild-type enzyme (Table 1). The catalytic parameters of variant L476Q concerning decarboxylation of benzoylformate are very similar to those of the wild-type enzyme, whereas the catalytic activity of the double mutant M365L-L461S is decreased to about 3% of the wild-type activity, with a tenfold higher K_m value and an increase in the K_i value (substrate inhibition constant).

Although the screening was performed with *ortho*-methylbenzaldehyde (**1 b**) as the only substrate, no benzoin **3** was detected when carboligation was performed in the presence of both purified BFD variants. However, quantitative formation of enantiopure (*S*)-2-hydroxy-1-(2-methylphenyl)-propanone ((*S*)-*o*-Me-2-HPP, **2 b**) was observed when excess acetaldehyde was added [Scheme 1 A]. These results indicate that both BFD variants are able to accept *ortho*-methylbenzaldehyde (**1 b**) as a donor but not as an acceptor substrate. Presumably, the *o*-2-

Table 1. *Carboligation[a] and decarboxylation activities[b] of the wild-type and mutant enzymes.*

Enzyme	Carboligation activity		Decarboxylation activity (benzoylformate)			Carboligation activity (20% DMSO)		Decarboxylation activity (20% DMSO) (benzoylformate)		
	spec. activity [U mg^{-1}]	*ee* (*S*)-2-HPP [%]	V_{max} [U mg^{-1}]	K_m [mM]	K_i [mM]	spec. activity [U mg^{-1}]	*ee* (*S*)-2-HPP [%]	V_{max} [U mg^{-1}]	K_m [mM]	K_i [mM]
wtBFD[c]	7.1 ± 0.7	83	327 ± 12	0.54 ± 0.09	143 ± 38	6.4 ± 0.7	82	327 ± 12	0.54 ± 0.1	143 ± 38
L476Q[c]	28.1 ± 2.5	95	246 ± 16	0.58 ± 0.07	100 ± 27	32.4 ± 1.7	93	246 ± 16	0.58 ± 0.1	100 ± 27
M365L-L461S						0.6 ± 0.2	93	10.6 ± 0.3	6.1 ± 0.5	18 ± 40

[a] Initial reaction velocities were measured with acetaldehyde (500 mM) and benzaldehyde (40 mM) as substrates at 30 °C in KPi buffer (50 mM, pH 7.0) containing Mg^{2+} (2.5 mM) and ThDP (0.5 mM), with or without DMSO [20% (v/v)] as a cosolvent as indicated, and with 50–300 µg enzyme. [b] Kinetic data were measured with benzoylformate (0.03–60 mM) as a substrate at 30 °C in KPi buffer (50 mM, pH 7.0), Mg^{2+} (2.5 mM), and ThDP (0.5 mM), with or without DMSO as a cosolvent [20% (v/v)] as indicated, and with 0.025 U enzyme. [c] Various kinetic data concerning wild-type BFD and L476Q have already been published.[8] K_m, Michaelis constant; K_i, inhibition constant; V_{max}, maximum reaction velocity.

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HPP derivative **2 b** was detected during the library screening because both compounds, the benzoin **3** and the *o*-Me-2-HPP derivative **2 b**, raise positive signals in the colorimetric assay at a concentration of 0.8 mM. Although acetaldehyde, which is necessary for this reaction, was not added to the reaction mixture during screening, it was probably generated by enzymatic oxidation of ethanol by cellular alcohol dehydrogenases in the crude extract. Ethanol was added to the screening mixture as a cosolvent for the aromatic aldehyde.[25]

Substrate and product range of the carboligation reaction

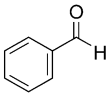
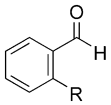
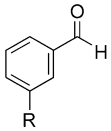
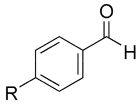
To investigate the substrate and product ranges of the BFD variants L476Q and M365L-L461S, reactions were carried out on a semipreparative scale with different substituted benzaldehyde derivatives, in the presence of acetaldehyde (Table 2). Both BFD variants converted all tested benzaldehyde derivatives, including *ortho*-substituted benzaldehydes, into the corresponding (*S*)-2-hydroxy ketones **2 a – p** with high to excellent enantioselectivities. With variant L476Q as a catalyst, quantitative conversion was observed on an analytical scale within 20 h reaction time, whereas with variant M365L-L461S conversion and enantioselectivity were shown to be significantly lower.

With the mutant enzyme BFD-L476Q, preparative batch processes were set up with *ortho*-substituted benzaldehyde substrates in the presence of excess acetaldehyde. The compounds **2 b – f** were thus prepared in an enantiopure form in 55 – 95 % yield. The absolute configuration of the 2-HPP derivatives **2** was determined to be (*S*) by comparison with the HPLC and optical rotation data of the corresponding (*R*)-enantiomers synthesized by BAL-catalyzed reaction.

Possible effects of the amino acid substitutions on the substrate range of the enzyme

The overall architecture of BFD[26] resembles that of other members of the ThDP-dependent enzyme family, such as pyruvate decarboxylase (PDC)[27] and pyruvate oxidase,[28] where cofactor- and metal-binding residues are conserved.[29] Interestingly, there is little similarity between the active-site residues when limited to those that are directly involved in the binding of the cofactors, ThDP and the Mg^{2+} ion.[26] The structural positions of the mutated residues of the selected mutant enzymes were identified by visual inspection of the 3D structure of the wild-type enzyme (Figure 1). Residue M365 of the double mutant M365L-L461S is located on the surface of the protein; the bulky hydrophobic residue L461 is located close to the cofactor ThDP and may interact with transition states and intermediates during the catalytic process. Thus, this leucine residue is likely to influence the substrate range of the acyl donor of the carboligation reaction. L461 in BFD from *Ps. putida* is probably located at the position equivalent to that of a glutamate residue that is conserved among all PDC sequences (E473 in *Zymomonas mobilis* (*Z.m.*) PDC, E477 in yeast PDC) as well as in indole-3-pyruvate decarboxylase from *Enterobacter cloacae* (E469).[30] Moreover, L461 exhibits positional homology to the hydrophobic side chain of I472 in *Z.m.* PDC (corresponding to I476 in yeast PDC), which has been shown to alter the substrate

Table 2. *Substrate and product range of wild-type BFD and the BFD variants L476Q and M365L-L461S.*[a]

acyl donor 1	R	No. 2[b]	wt BFD[c] conv. [%]	wt BFD[c] *ee* [%]	L476Q conv. [%]	L476Q *ee* [%]	M365L-L461S conv. [%]	M365L-L461S *ee* [%]
		a	99	92	100	97.5	97	94.5
	Me	b	4	n.d.[d]	100	> 99	83	98
	OMe	c	0		97	> 99	46	> 99
	F	d	91	89	100	> 99	100	98
	Cl	e	0		100	> 99	85	> 99
	Br	f	0		98	> 99	90	> 99
	Me	g	99	97	100	99	100	99
	OMe	h	94	96	100	99	100	99
	F	i	100	87	100	> 99	94	> 99
	Cl	j	94	94	100	97	100	98
	Br	k	68	96	97	98	100	98.5
	Me	L	65	88	100	98	98	98
	OMe	m	23	92	100	> 99	67	42
	F	n	69	87	100	97	87	97
	Cl	o	85	82	100	96.5	100	95.5
	Br	p	42	83	100	96.5	100	96

[a] Reactions catalyzed by wild-type BFD were all performed on an analytical scale at 30 °C by batch synthesis in KPi buffer (50 mM, pH 7.0) containing Mg^{2+} (2.5 mM), ThDP (0.5 mM), and lyophilized enzyme (0.2 mg mL^{-1}) and were stopped after 20 h. Reactions catalyzed by the BFD variants were performed at 30 °C by batch synthesis in the same buffer with DMSO [20 (v/v)] added and were stopped after 20 – 48 h. The absolute configuration of the product was determined to be (*S*) by comparison of HPLC analysis data with literature values[17] or with analytical data for the (*R*)-HPP derivatives.[23] [b] The compounds **2 a**, **g – p** were synthesized on an analytical scale. [c] The presented experimental data concerning wild-type BFD have already been published (without DMSO, 10 mM substrate **1**).[17] [d] Not determined.

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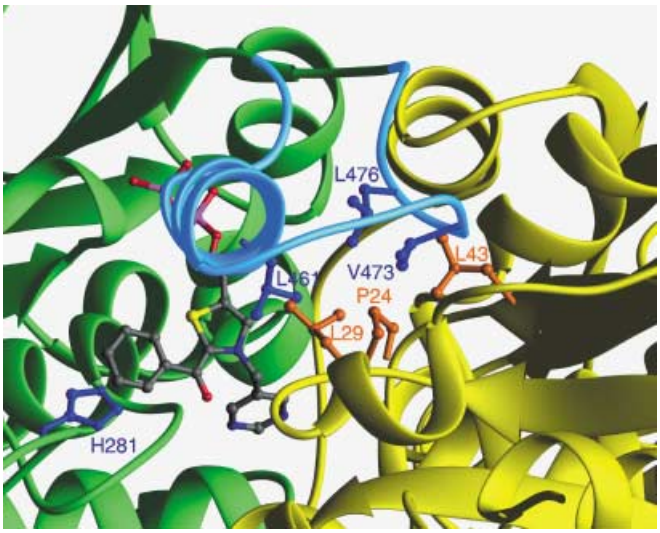

Figure 1. Partial view into the 3D structure of wild-type BFD showing one active center at the interface of two monomers (green and yellow). The substrate benzaldehyde bound to the cofactor ThDP is shown in grey. Amino acid residues shown in blue correspond to those forming a loop near the active center. Residues shown in orange belong to the yellow domain and are in direct contact with the blue residues.

specificity of *Z.m.* PDC.[31] The reduction of the side chain size at position 461 and the resulting enlarged substrate binding site could facilitate the initial binding of *ortho*-substituted benzaldehyde derivatives to the C-2 atom of ThDP. Besides, the serine residue that is introduced at position 461 in the mutant and replaces the hydrophobic leucine residue is hydrophilic and able to form hydrogen bonds, which could also influence the binding of substrates.

Additionally, residue L461 could affect the binding of the cofactor ThDP through the neighbouring residue L460, which is involved in cofactor binding.[26] L461 seems to be important for the binding of the substrate since a decrease in the V_{max} value as well as an increase in the K_m value was observed for the decarboxylation of benzoylformate by the mutant M365L-L461S compared to that with the wild-type enzyme, whereas these catalytic parameters are not effected in the case of the variant L476Q (Table 1).

L476 is part of a loop that covers the active center of the enzyme like a lid (Figure 1). Mutation at this position could increase the flexibility of this lid and lead to a faster entry of the substrates. In fact, the influence of the L476Q exchange on the substrate specificity of the carboligation reaction seems to be more general. In addition, this residue was found to be a hot spot concerning carboligation activity. In a screening concerning progression of the activity of the side reaction of the enzyme in the presence of water-miscible organic solvents, several variants with modifications at this position were shown to have a higher carboligation activity than the wild-type enzyme.[8] Mutant L476Q showed complete conversion of all substituted benzaldehyde derivatives in the presence of acetaldehyde within 20 h (preparative scale: 20-48 h), whereas with the mutant M365L-L461S total conversion was not achievable under similar conditions.

Discussion and Conclusions

Herein, we present two BFD variants that accept *ortho*-substituted benzaldehyde derivatives as donor substrates, which means that these benzaldehyde derivatives are bound to the cofactor ThDP and are then ligated to the acyl acceptor acetaldehyde. As was deduced from the 3D structure of the wild-type enzyme, L461 in the double mutant (BFD M365L-L461S) and L476 in the single mutant (BFD L476Q) are not directly involved in the binding of the acyl donor (Figure 1) but both mutations enable the binding of *ortho*-substituted benzaldehyde derivatives to the cofactor ThDP. Analysis of the 3D structure of these BFD variants could give interesting insights into the reaction mechanism of ThDP-dependent enzymes.

Since BFD and BAL are enantiocomplementary with regard to 2-HPP formation,[32] most 2-HPP and benzoin derivatives can be synthesized in either enantiomeric form by using BFD and BAL wild-type enzymes as well as the newly developed BFD muteins. Together with PDC, these enzymes and mutant enzymes provide a toolbox for the enantioselective synthesis of 2-hydroxy ketones as chiral building blocks for organic synthesis.

Recently, the mutant BFD-H281A, generated by rational protein design,[33] was recognized as a potent catalyst to perform an asymmetric cross-benzoin condensation by starting from benzaldehyde and *ortho*-substituted benzaldehyde derivatives as substrates.[34] *ortho*-Substituted benzaldehyde derivatives were accepted selectively by this enzyme as acceptor but not as donor substrates.

Experimental procedures

General: All reagents were purchased from Fluka (Taufkirchen, Germany) or Sigma (Taufkirchen, Germany) unless otherwise stated. Enzymes for recombinant DNA work were purchased from New England Biolabs (Schwalbach, Germany), Roche Molecular Biochemicals (Mannheim, Germany) or Gibco BRL (Karlsruhe, Germany). For affinity chromatography of the BFD variants, Ni-nitrilotriacetate (NTA)-agarose (Qiagen, Hilden, Germany) was used. Oligonucleotide primers were obtained from MWG Biotech (Ebersberg, Germany). The pKK 233-2 expression plasmid was purchased from Clontech (Palo Alto, CA, USA) and the *E. coli* K 12 strain SG 13009 from Qiagen (Hilden, Germany).

NMR spectra were recorded on a Bruker AMX 300 spectrometer. Chemical shifts δ are reported in ppm relative to $CHCl_3$ (^{1}H: $\delta = 7.27$ ppm) and $CDCl_3$ (^{13}C: $\delta = 77.23$ ppm) as internal standards. Flash column chromatography was conducted on silica gel 60 (40 – 63 μm). TLC was carried out on aluminium sheets precoated with silica gel $60F_{254}$ (Merck). Enantiomeric excesses were determined by HPLC analysis by using a Hewlett Packard 1100 Series HPLC system equipped with a diode-array detector and with a Daicel Chiralcel OB or a Chiralcel OD-H column (250 × 4 mm). GC – MS spectra were determined on a Hewlett Packard 6890 Series GC System and a Hewlett Packard 5973 mass-selective detector; capillary column HP-5MS (5 % phenylmethylsiloxane, 30 m, 250 μm; T_{GC}(injector) = 250 °C, T_{MS}(ion source) = 200 °C, time program (oven): $T_{0min} = 60$ °C, $T_{3min} = 60$ °C, $T_{14min} = 280$ °C (heating rate 20 °C min^{-1}), $T_{19min} = 280$ °C; MS: EI, 70 eV). Optical rotations were measured with a Jasco P-1020 polarimeter. M.p.s were measured on a Büchi B-540 capillary tube apparatus. UV spectra were recorded with a Ultrospec 2000 UV/Vis

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spectrophotometer from Pharmacia Biotech, Sweden. Microanalyses were carried out at the Analytical Department, Chemische Institute der Universität Bonn, Germany.

Construction and screening of a BFD random mutagenesis library: A BFD random mutagenesis library was constructed as described in reference [8] by using the wild-type BFD gene with a region coding for a hexahistidine tag as a template.

For the screening, masterplates were replicated into 96-well plates containing LB medium (100 μL) supplemented with ampicillin (100 μg mL^{-1}) and neomycin (25 μg mL^{-1}) and incubated for 20 h at 37 °C. An aliquot of each culture was transferred into 96-deep-well plates containing LB medium (1.2 mL) supplemented with ampicillin (100 μg mL^{-1}) and neomycin (25 μg mL^{-1}). After incubation at 37 °C for 4 h, protein expression was induced by addition of isopropyl-β-D-thiogalactoside (IPTG, 1 mM). The plates were incubated at 37 °C for 16 h. Cells were harvested by centrifugation (15 min, 4000 rpm). The cell pellets were dispersed in potassium phosphate buffer (100 μL; 50 mM, pH 7.0) containing ThDP (0.5 mM) and $MgSO_4$ (2.5 mM); cell lysis was achieved by addition of lysozyme (1 mg mL^{-1}) and incubation at 30 °C for 30 min on a shaker. The crude extracts were transferred into two fresh 96-well plates and 2-fold substrate solution in potassium phosphate buffer (100 μL; 50 mM, pH 7.0) containing ethanol (1.5 M), ThDP (0.5 mM), and $MgSO_4$ (2.5 mM). 2-Methylbenzaldehyde (50 mM) was added to each well. The reaction mixture was incubated at 30 °C for 18 h. Afterwards, the 96-well plates were centrifuged (15 min, 4000 rpm, RT) and the supernatant (100 μL) was transferred into new 96-well plates and assayed for the accumulation of condensation products with the colorimetric assay described below.[8, 24] To each well containing supernatant of the reaction mixture, NaOH (10 μL; 3 mM) and 2,3,5,-triphenyltetrazolium chloride (10 μL; 0.4 % (w/v) in methanol) were added and the formation of the red formazan dye was monitored at 510 nm.

Expression and purification of selected BFDHis mutants: Expression and purification of wild-type (wt)BFDHis and the BFDHis mutants was performed as described previously for PDC[31] by using potassium phosphate buffer (50 mM, pH 7.0) for the Ni-NTA-chromatography and potassium phosphate buffer (50 mM, pH 6.0) containing ThDP (0.5 mM) and $MgSO_4$ (2.5 mM) as elution buffer for the subsequent gel chromatography. Lyophilized BFDHis mutants, stored at −20 °C, were stable for several months. Determination of the protein concentration was performed according to Bradford[35] by using bovine serum albumin for calibration.

Assay for the determination of the decarboxylation activity: The activities of the enzymes concerning the decarboxylation of benzoylformate were determined by using a coupled enzymatic test, as described elsewhere.[17]

Computational graphic studies: The structural models of the different mutations were constructed based on the crystal structure of wild-type BFD (pdb entry: 1BFD),[26] by using the programs WHATIF and Ribbons[36] running on a Silicon Graphics workstation.

Enzymatic synthesis and analysis of the 2-hydroxy ketones 2: Enzymatic syntheses were performed in potassium phosphate buffer (50 mM, pH 7.0) containing $MgSO_4$ (2.5 mM), ThDP (0.5 mM), DMSO (20 % (v/v)), acetaldehyde (500 mM), and benzaldehyde (40 mM) or the respective *ortho*-substituted benzaldehyde derivative (25 mM) as well as lyophilized enzyme (2.0 mg mL^{-1}, preparative scale: total volume 10 mL). The reaction mixture was incubated at 30 °C. Extraction of the reaction mixture with dichloromethane (preparative scale: ethyl acetate) followed by phase separation by centrifugation (13 000 rpm) was carried out after the conversion remained static (20 – 48 h). The solvent was removed under reduced pressure. Then the compounds (*S*)-**2 b – f** synthesized on a preparative scale were purified by column chromatography (isohexane/ethyl acetate 5:1). All products were analyzed by GC – MS and chiral phase HPLC. Products synthesized on a preparative scale were additionally characterized by NMR spectroscopy. As a standard, compounds (*R*)-**2 b – f** were synthesized by BAL-catalyzed transformation according to ref. [23].

HPLC and GC – MS data of the compounds **2 a** and **2 f – p** have already been reported in the literature.[17]

(*S*)-2-Hydroxy-1-(2-methylphenyl)propan-1-one [(*S*)-2 b]: Yellow oil; yield: 28 mg (69 %); *ee* > 99 %; $[\alpha]_D^{25} = -58.3°$ ($c = 1.0$, $CHCl_3$); HPLC (Chiralcel OD-H; isohexane/2-propanol 99:1, 0.5 mL min^{-1}, 40 °C) (*S*) 28.23 min; ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 1.32$ (d, $J = 7.1$ Hz, 3 H), 2.50 (s, 3 H), 5.07 (q, $J = 7.1$ Hz, 1 H), 7.30 (d, $J = 8.5$ Hz, 2 H), 7.43 (t, $J = 7.5$ Hz, 1 H), 7.53 (d, $J = 7.8$ Hz, 1 H) ppm; ^{13}C NMR (75.4 MHz, $CDCl_3$): $\delta = 21.12$, 21.16, 70.9, 126.0, 128.5, 132.2, 132.3, 134.4, 139.1, 205.9 ppm; GC – MS: $R_t = 8.77$ min; EI-MS (70 eV): *m/z* (%): 164 (2), 149 (1), 121 (12), 119 (100), 91 (62), 77 (5), 65 (19).

(*R*)-2 b: $[\alpha]_D^{25} = 51.4°$ ($c = 1.0$, $CHCl_3$); *ee* > 99 %; HPLC (Chiralcel OD-H; isohexane/2-propanol 99:1, 0.5 mL min^{-1}, 40 °C) (*R*) 29.78 min.

(*S*)-2-Hydroxy-1-(2-methoxyphenyl)propan-1-one [(*S*)-2 c]: Yellow oil; yield: 43 mg (95 %); *ee* > 99 %; $[\alpha]_D^{25} = -115.8$ ($c = 1.0$, $CHCl_3$); HPLC (Chiralcel OB; isohexane/2-propanol 98:2, 0.75 mL min^{-1}, 20 °C) (*S*) 34.80 min; ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 1.33$ (d, $J = 7.0$ Hz, 3 H), 3.91 (s, 3 H), 5.15 (q, $J = 7.0$ Hz, 1 H), 6.98 (d, $J = 8.4$ Hz, 1 H), 7.04 (t, $J = 7.5$ Hz, 1 H), 7.52 (td, $J = 7.8$, 1.8 Hz, 1 H), 7.79 (dd, $J = 7.8$, 1.8 Hz, 1 H) ppm; ^{13}C NMR (75.4 MHz, $CDCl_3$): $\delta = 20.6$, 55.7, 73.1, 111.8, 121.2, 124.4, 131.3, 134.8, 158.8, 204.1 ppm; GC – MS: $R_t = 9.92$ min; EI-MS (70 eV): *m/z* (%): 180 (1), 165 (1), 137 (13), 135 (100), 107 (8), 92 (13), 77 (30).

(*R*)-2 c: $[\alpha]_D^{25} = 108.0$ ($c = 1.0$, $CHCl_3$); *ee* > 99 %; HPLC (Chiralcel OB; isohexane/2-propanol 98:2, 0.75 mL min^{-1}, 20 °C; (*R*) 46.07 min.

(*S*)-1-(2-Fluorophenyl)-2-hydroxy-propan-1-one [(*S*)-2 d]: Yellow oil; yield: 27 mg (64 %); *ee* > 99 %; $[\alpha]_D^{25} = -66.3$ ($c = 1.0$, $CHCl_3$); HPLC (Chiralcel OB; *n*-heptane/2-propanol 90:10, 0.75 mL min^{-1}, 25 °C) (*S*) 11.33 min; ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 1.41$ (dd, $J = 7.1$, 1.4 Hz, 3 H), 3.79 (br s, 1 H), 5.05 (qd, $J = 7.1$, 2.7 Hz, 1 H), 7.17 (ddd, $J = 11.1$, 8.4, 1.0 Hz, 1 H), 7.29 (td, $J = 7.5$, 1.0 Hz, 1 H), 7.56-7.64 (m, 1 H), 7.94 (td, $J = 7.5$, 1.8 Hz, 1 H) ppm; ^{13}C NMR (75.4 MHz, $CDCl_3$): $\delta = 20.9$, 73.0 (d, 9.3 Hz), 117.0 (d, 23.5 Hz), 122.4 (d, 13.5 Hz), 125.1 (d, 3.2 Hz), 131.3 (d, 2.9 Hz), 135.8 (d, 9.2 Hz), 161.8 (d, 255.2 Hz), 201.1 (d, 4.3 Hz) ppm; GC – MS: $R_t = 8.12$ min; EI-MS: (70 eV): *m/z* (%): 168, 153 (1), 125 (31), 123 (100), 97 (9), 95 (23), 75 (13).

(*R*)-2 d: $[\alpha]_D^{25} = 59.7$ ($c = 1.0$, $CHCl_3$); *ee* > 99 %; HPLC (Chiralcel OB; *n*-heptane/2-propanol 90:10, 0.75 mL min^{-1}, 25 °C) (*R*) 15.18 min.

(*S*)-1-(2-Chlorophenyl)-2-hydroxy-propan-1-one [(*S*)-2 e]: Yellow oil, yield: 38 mg (82 %), *ee* > 99 %; $[\alpha]_D^{25} = -81.8$ ($c = 1.0$, $CHCl_3$); HPLC (Chiralcel OB, isohexane/2-propanol = 98:2, 0.75 mL min^{-1}, 20 °C) (*S*) 28.43 min; ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 1.33$ (d, $J = 7.2$ Hz, 3 H), 3.69 (br s, 1 H), 5.10 (q, $J = 7.2$ Hz, 1 H), 7.33-7.41 (m, 1 H), 7.44 – 7.47 (m, 3 H) ppm; ^{13}C NMR (75.4 MHz, $CDCl_3$): $\delta = 20.2$, 72.4, 127.2, 129.3, 131.0, 131.6, 132.6, 136.0, 205.1 ppm; GC – MS: $R_t = 9.33$ min, EI-MS (70 eV): *m/z* (%): 184, 149 (8), 141 (73), 139 (100), 113 (14), 111 (23), 77 (25), 51 (6); HR-MS: *m/z*: calcd. for $C_9H_9O_2Cl$ $[M]^+$: 184.0288; found: 184.0291.

(*R*)-2 e: $[\alpha]_D^{25} = 86.6$ ($c = 1.0$, $CHCl_3$); *ee* > 99 %; HPLC (Chiralcel OB, isohexane/2-propanol = 98:2, 0.75 mL min^{-1}, 20 °C) (*R*) 34.56 min.

(*S*)-1-(2-Bromophenyl)-2-hydroxy-propanone [(*S*)-2 f]: Yellow oil; yield: 32 mg (55 %); *ee* > 99 %; $[\alpha]_D^{25} = -64.1$ ($c = 1.0$, $CHCl_3$); HPLC (Chiralcel OB; isohexane/2-propanol 90:10, 0.75 mL min^{-1}, 25 °C) (*S*) 14.70 min; ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 1.34$ (d, $J = 7.2$ Hz, 3 H), 5.06 (q, $J = 7.2$ Hz, 1 H), 7.35 – 7.41 (m, 3 H), 7.66 (dd, $J = 7.9$, 1.5 Hz,

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1 H) ppm; ^{13}C NMR (75.4 MHz, $CDCl_3$): $\delta = 20.1$, 72.2, 119.6, 127.6, 129.0, 132.5, 134.2, 138.3, 205.8 ppm; GC – MS: $R_t = 9.93$ min; EI-MS (70 eV): *m/z* (%): 187 (26), 185 (100), 183 (70), 159 (5), 157 (26), 155 (22), 149 (13), 105 (9), 77 (46).

(*R*)-2 f: $[\alpha]_D^{25} = 57.6$ ($c = 1.0$, $CHCl_3$); *ee* > 99 %; HPLC (Chiralcel OB, isohexane/2-propanol = 90:10, 0.75 mL min^{-1}, 20 °C) (*R*) 15.95 min.

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[1] B. Schulze, M. G. Wubbolts, *Curr. Opin. Biotechnol*. **1999**, *10*, 609 – 615.
[2] K. Faber, *Biotransformations in Organic Chemistry*, 4th Ed, Springer, Berlin, **2000**.
[3] *Enzyme Catalysis in Organic Synthesis, Vol. I – III*, 2nd Ed (Eds.: K. Drauz, H. Waldmann), Wiley-VCH, Weinheim, **2002**.
[4] a) M. Kikuchi, K. Ohnishi, S. Harayama, *Gene* **2000**, *243*, 133 – 137; b) S. Lutz, S. J. Benkovic, *Curr. Opin. Biotechnol*. **2000**, *11*, 319 – 324; c) M. Ostermeier, A. E. Nixon, S. J. Benkovic, *Bioorg. Med. Chem.* **1999**, *7*, 2139 – 2144; d) R. K. Saiki, D. H. Gelfand, S. Stoffel, S. J. Scharf, R. Higuchi, G. T. Horn, K. B. Mullis, H. A. Erlich, *Science* **1988**, *239*, 487 – 491; e) Z. Shao, H. Zhao, L. Giver, F. H. Arnold, *Nucleic Acids Res.* **1998**, *26*, 681 – 683; f) W. P. C. Stemmer, *Nature* **1994**, *370*, 389 – 391; g) W. P. C. Stemmer, *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91*, 10 747 – 10 751; h) A. A. Volkov, Z. Shao, F. H. Arnold, *Nucleic Acids Res.* **1999**, *27*, e18; i) H. Zhao, F. H. Arnold, *Nucleic Acids Res.* **1997**, *25*, 1307 – 1308.
[5] S. V. Taylor, P. Kast, D. Hilvert, *Angew. Chem.* **2001**, *113*, 3408 – 3436; *Angew. Chem. Int. Ed.* **2001**, *40*, 3310 – 3335.
[6] H. Zhao, F. H. Arnold, *Protein Eng*. **1999**, *12*, 47 – 53.
[7] L. Giver, A. Gershenson, P.-O. Freskgard, F. H. Arnold, *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 12 809 – 12 813.
[8] B. Lingen, J. Grötzinger, D. Kolter, M.-R. Kula, M. Pohl, *Protein Eng.* **2002**, *15*, 585 – 593.
[9] J. C. Moore, F. H. Arnold, *Nat. Biotechnol.* **1996**, *14*, 458 – 467.
[10] L. You, F. H. Arnold, *Protein Eng.* **1994**, *9*, 77 – 83.
[11] a) M. T. Reetz, S. Wilensek, D. Zha, K.-E. Jäger, *Angew. Chem.* **2001**, *113*, 3701 – 3703; *Angew. Chem. Int. Ed.* **2001**, *40*, 3589 – 3591; b) D. Zha, S. Wilensek, M. Hermes, K.-E. Jäger, M. T. Reetz, *Chem. Commun.* **2001**, 2664 – 2665.
[12] O. May, P. T. Nguyen, F. H. Arnold, *Nat. Biotechnol*. **2000**, *18*, 317 – 320.
[13] a) P. Siegert, H. Iding, M. Baumann, M. J. McLeish, G. Kenyon, M. Pohl, *Proceedings of the 4th International Congress on Biochemical Engineering*, **2000**, Stuttgart, Germany; b) P. Siegert, H. Iding, M. Baumann, M. McLeish, G. Kenyon, M. Pohl, unpublished work.
[14] M. M. Altamirano, J. M. Blackburn, C. Aguayo, A. R. Fersht, *Nature* **2000**, *403*, 617 – 622.
[15] Y. Takato, O. Shinya, K. Hiroyuki, *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 5511 – 5515.
[16] J.-H. Zhang, G. Dawes, W. P. C. Stemmer, *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 4504 – 4509.
[17] H. Iding, T. Dünnwald, L. Greiner, A. Liese, M. Müller, P. Siegert, J. Grötzinger, A. S. Demir, M. Pohl, *Chem. Eur. J.* **2000**, *6*, 1483 – 1495.
[18] T. Dünnwald, A. S. Demir, P. Siegert, M. Pohl, M. Müller, *Eur. J. Org. Chem*. **2000**, 2161 – 2170.
[19] a) M. Pohl, *Adv. Biochem. Eng. Biotechnol*. **1997**, *58*, 15 – 43; b) U. Schörken, G. A. Sprenger, *Biochim. Biophys. Acta* **1998**, *1385*, 229 – 243; c) G. A. Sprenger, M. Pohl, *J. Mol. Catal. B: Enzym.* **1999**, *6*, 145 – 159; d) M. Pohl, B. Lingen, M. Müller, *Chem. Eur. J.* **2002**, *8*, 5288 – 5295.
[20] A. S. Demir, T. Dünnwald, H. Iding, M. Pohl, M. Müller, *Tetrahedron: Asymmetry* **1999**, *10*, 4769 – 4774.
[21] H. Iding, dissertation **1998**, University of Düsseldorf.
[22] T. Dünnwald, M. Müller, *J. Org. Chem.* **2000**, *65*, 8608 – 8612.
[23] a) A. S. Demir, M. Pohl, E. Janzen, M. Müller, *J. Chem. Soc. Perkin Trans. 1* **2001**, 633 – 635; b) A. S. Demir, Ö. Şeşenoglu, E. Eren, B. Hosrik, M. Pohl, E. Janzen, D. Kolter, R. Feldmann, P. Dünkelmann, M. Müller, *Adv. Synth. Catal*. **2002**, *344*, 96 – 103.
[24] M. Breuer, M. Pohl, B. Hauer, B. Lingen, *Anal. Bioanal. Chem*. **2002**, *374*, 1069 – 1073.
[25] When crude cell lysate (BFD-L476Q) was used without adding acetaldehyde, formation of **2 b** and its tautomer was observed in the presence of ethanol, whereas no product could be determined (GC – MS) in the presence of DMSO instead of ethanol, or without added cosolvent.
[26] M. S. Hasson, A. Muscate, M. J. McLeish, L. S. Polovnikova, J. A. Gerlt, G. L. Kenyon, G. A. Petsko, D. Ringe, *Biochemistry* **1998**, *28*, 9918 – 9930.
[27] D. Dobritzsch, S. König, G. Schneider, G. G. Lu, *J. Biol. Chem.* **1998**, *273*, 20196 – 20204.
[28] a) Y. A. Muller, G. E. Schulz, *Science* **1993**, *259*, 965 – 967; b) Y. A. Muller, G. Schumacher, R. Rudolph, G. E. Schulz, *J. Mol. Biol.* **1994**, *237*, 315 – 335.
[29] For a recent review on the mechanism of ThDP-dependent enzymes, see: F. Jordan, *Nat. Prod. Rep.* **2003**, *20*, 184 – 201.
[30] J. Koga, T. Adachi, H. Hidaka, *Mol. Gen. Genet.* **1991**, *226*, 1 – 6.
[31] M. Pohl, P. Siegert, K. Mesch, H. Bruhn, J. Grötzinger, *Eur. J. Biochem.* **1998**, *257*, 538 – 546.
[32] In general, in BAL-catalyzed biotransformations *ortho*-substituted aromatic aldehydes were transformed with lower conversion rates than the *meta*- and *para*-substituted aldehydes; see also ref. [23].
[33] L. S. Polovnikova , M. J. McLeish, E. A. Sergienko, J. T. Burgner, N. L. Anderson, A. K. Bera, F. Jordan, G. L. Kenyon, M. S. Hasson, *Biochemistry* **2003**, *42*, 1820 – 1830.
[34] P. Dünkelmann, D. Kolter-Jung, A. Nitsche, A. S. Demir, P. Siegert, B. Lingen, M. Baumann, M. Pohl, M. Müller, *J. Am. Chem. Soc.* **2002**, *124*, 12084 – 12085.
[35] M. M. Bradford, *Anal. Biochem.* **1976**, *72*, 248 – 254.
[36] M. Carson, *J. Appl. Crystallogr.* **1991**, *24*, 946 – 950.

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