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Improving the carboligase activity of benzoylformate decarboxylase from *Pseudomonas putida* by a combination of directed evolution and site-directed mutagenesis

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Benzoylformate decarboxylase (BFD) from *Pseudomonas putida* was subjected to directed molecular evolution to generate mutants with increased carboligase activity which is a side reaction of the enzyme. After a single round of random mutagenesis mutants were isolated which exhibited a 5-fold increased carboligase activity in aqueous buffer compared to the wild-type enzyme with a high enantiomeric excess of the product (*S*)-2-hydroxy-1-phenyl-propanone. From the same library, mutants with enhanced carboligase activity in water-miscible organic solvents have been isolated. The selected mutants have been characterized by sequencing, revealing that all mutants carry a mutation at Leu476, which is close to the active site but does not directly interact with the active center. BFD-L476Q has a 5-fold higher carboligase activity than the wild-type enzyme. L476 was subjected to saturation mutagenesis yielding eight different mutants with up to 5-fold increased carboligase activity. Surprisingly, all L476 mutants catalyze the formation of 2-hydroxy-1-phenyl-propanone with significantly higher enantioselectivity than the wild-type enzyme although enantioselectivity was not a selection parameter. Leu476 potentially plays the role of a gatekeeper of the active site of BFD, possibly by controlling the release of the product. The biocatalyst could be significantly improved for its side reaction, the C–C bond formation and for application under conditions that are not optimized in nature.

Keywords: benzoylformate decarboxylase/directed evolution/organic solvents/random mutagenesis/ThDP/total activity

Introduction

The use of enzymes in bioorganic chemistry is now widely recognized as a versatile alternative to traditional organic chemistry (Azerad, 1995; Faber, 1997; Roberts, 1998; Schulze and Wubbolts, 1999; Koeller and Wong, 2001). Because of their regio- and stereoselectivity enzymes are particularly valuable to obtain enantiomerically pure intermediates and products (Patel, 2000).

Enzymes which catalyze C–C bond formation such as aldolases, transketolases, oxynitrilases (Fessner and Walter, 1996; Seoane, 2000) and thiamin-diphosphate (ThDP)-dependent enzymes (Pohl, 1997; Iding *et al.*, 1998; Schellenberger, 1998; Schörken and Sprenger, 1998; Sprenger and Pohl, 1999; Ward and Baev, 2000; Ward and Singh, 2000) are of great importance in bioorganic chemistry.

ThDP-dependent benzoylformate decarboxylase (BFD, EC 4.1.1.7, accession no. J05293) from *Pseudomonas putida* is involved in the degradation of aromatic compounds of the mandelate catabolism (Gunsalus *et al.*, 1953a,b; Stanier *et al.*, 1953). The BFD gene has been cloned (Tsou *et al.*, 1990) and the crystal structure solved (Hasson *et al.*, 1998). The main enzymatic reaction catalyzed by BFD is the non-oxidative decarboxylation of benzoylformate to benzaldehyde. Additionally the enzyme catalyzes the enantioselective formation of C–C bonds (Wilcocks *et al.*, 1992) as a side reaction with yet unknown physiological role. Due to this carboligase activity BFD has recently been introduced as a catalyst for the enantioselective formation of (*S*)-2-hydroxy-1-phenylpropan-1-one [(*S*)-2-HPP; Figure 1] from benzaldehyde and acetaldehyde (Iding *et al.*, 2000) as well as for the enantioselective formation of other asymmetric 2-hydroxy ketones (Dünnwald *et al.*, 2000), enantiomerically pure (*R*)-benzoin and benzoin derivatives (Demir *et al.*, 1999) starting from different donor and acceptor aldehydes. The main problem concerning the application of substituted benzaldehyde derivatives as adducts for the formation of 2-hydroxy ketones is the low solubility of many of these compounds in aqueous buffer solution. Therefore, a catalytic process in the presence of organic solvents is of interest to increase the solubility of the aldehyde substrates and to facilitate the recovery of products. However, enzymes which are optimized for biological systems often show low stability and catalytic activity in non-natural environments (Arnold, 1993). In the case of BFD the wild-type enzyme is sufficiently stable in the presence of organic solvents, but the side reaction of the enzyme shall be synthetically used, which is characterized by a significantly lower catalytic activity than the main reaction. The use of enzymes in technical processes requires catalytic activities as high as possible for optimal productivity; therefore, BFD variants with enhanced carboligase activity in the presence of organic solvents would be useful biocatalysts.

Directed evolution has rapidly emerged to be the method of choice for the development and selection of mutated enzymes with improved properties (Bornscheuer and Pohl, 2001) such as improved thermostability (Giver *et al.*, 1998), altered substrate specificity (Matsumura and Ellington, 2001), increased enantioselectivity (Liebeton *et al.*, 2000), inverted enantioselectivity (May *et al.*, 2000), or improved activity in organic solvents (Chen and Arnold, 1991; Economou *et al.*, 1992; Moore and Arnold, 1996; You and Arnold, 1996; Song and Rhee, 2001). Thus, enzymes can be tailored for special demands of manufacturing processes for the production of intermediates of natural or pharmaceutical products without detailed knowledge of protein structure or structure–function relationships. As the crystal structure of BFD from *Ps.putida* is known (Hasson *et al.*, 1998), investigation of the structural basis of the newly introduced mutations is facilitated.

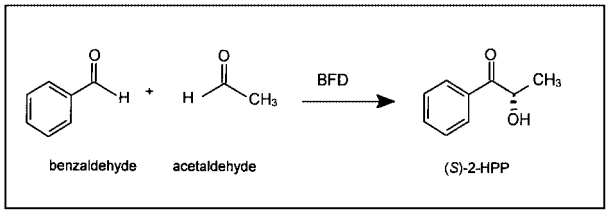

Fig. 1. Formation of (*S*)-2-HPP starting from aldehydes via BFD-catalyzed C–C bond formation.

Our goal was to obtain improved BFD muteins with enhanced carboligase activity in water-miscible organic solvents by directed evolution. We established a screening system in which the carboligase activity of the enzyme was monitored by a rapid colorimetric assay. Here we demonstrate that by using this system BFD mutants with increased carboligase activity in aqueous buffer and mutants with enhanced carboligase activity in water-miscible organic solvents were generated. In the highly active mutants, Leu476, which is not located in the active center of the enzyme, has been mutated.

Leu476 revealed to be a hot spot region for carboligase activity and has been investigated further by saturation mutagenesis which was shown to enhance the effectiveness of directed evolution (Miyazaki and Arnold, 1999; Liebeton *et al.*, 2000). This approach resulted in muteins with different residues at this position with up to 5-fold increased carboligase activity and higher enantioselectivity compared to the BFD wild-type enzyme.

Materials and methods

Reagents

Chemicals used in this work were purchased from Fluka (Taufkirchen, Germany) or Sigma (Taufkirchen). 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 BFD variants, Ni-NTA-agarose (Qiagen, Hilden, Germany) was used. Oligonucleotide primers were obtained from MWG Biotech (Ebersberg, Germany).

Generation of plasmid libraries of the BFD gene

The BFDHis gene was amplified by error-prone PCR (epPCR) in the presence of 0.2 mM dATP and 1 mM dGTP, dCTP, dTTP each, with 5 U *Taq* DNA polymerase (Gibco BRL, Karlsruhe, Germany) and varying concentrations of $MgCl_2$ and $MnCl_2$ (Table I) to influence the fidelity of the *Taq* DNA polymerase. The plasmid pKK233-2/BFDHis (Iding *et al.*, 2000) was used as template and the oligonucleotides pKK233-2 for (5′ CACACAGGAAACAGACCA-TGG 3′) and pKK233-2 rev (5′ TCCGCCAAAACAGCCAAGCTT 3′) as 5′- and 3′-primers, which are complementary to the vector regions immediately flanking the *Nco*I and *Hin*dIII sites, respectively. The following amplification conditions were applied: step 1: 95°C, 1 min; step 2: 50°C, 1 min; step 3: 72°C, 1 min; step 4: 72°C, 5 min. Steps 1–3 were repeated 30 times. Each PCR reaction was digested with *Dpn*I at 37°C for 2 h to remove methylated wild-type DNA. Fragments of 1.6 kb were isolated by extraction from an agarose gel, purified and digested with *Nco*I and *Hin*dIII at 37°C for 2 h. The digested fragments were purified and ligated into the *Nco*I and *Hin*dIII digested expression vector pKK233-2 (Clontech, Palo Alto CA, USA).

Table I. Conditions for epPCR used for generation of BFDHis mutant libraries

epPCR	$MgCl_2$ (mM)	$MnCl_2$ (mM)	Clones with carboligase activity (%)
1	1.5	–	96
2	7.0	–	90
3	1.5	0.5	47
4	7.0	0.5	26

$MgCl_2$ and $MnCl_2$ concentrations were altered to influence the fidelity of the *Taq* DNA polymerase. Each epPCR was performed with the wtBFDHis gene as template, oligonucleotides pKK233-2 for and pKK233-2 rev as primers, 0.2 mM dATP and 1 mM dGTP, dCTP, dTTP, each with 5 U *Taq* DNA polymerase. Four hundred independent clones of each library were screened for carboligase activity. Frequency of inactive clones is related to the point mutagenesis rate.

The *Escherichia coli* strain SG13009/pREP4 (Qiagen) was transformed with one of the ligation mixtures each according to Inoue *et al.* (Inoue *et al.*, 1990), plated on LB agar plates containing 100 μg/ml ampicillin and 25 μg/ml neomycin and incubated at 37°C. A total of 4×10^2 independent clones of each PCR reaction were transferred into 96-well plates and screened for carboligase activity. A total of 4×10^3 independent clones of *E.coli* transformed with the ligation mixture of PCR condition 3 (Table I: 1.5 mM $MgCl_2$, 0.5 mM $MnCl_2$) were transferred into 96-well plates. After 20 h growth at 37°C these masterplate cultures were stored with 10% glycerol at –80°C.

Construction of BFDHis variants with single amino acid exchanges

The two mutants with a single amino acid exchange corresponding to each of the two amino acid substitutions found in BFD-S181T-L476PHis were constructed. The plasmid DNA pKK233-2/BFD-S181T-L476PHis was digested with *Nco*I and *Nhe*I and the 920 bp fragment carrying the mutation **T**CA → **A**CA (S181T) was ligated into the *Nco*I/*Nhe*I digested vector pKK233-2/BFDHis lacking the corresponding fragment, leading to the single mutant plasmid pKK233-2/BFD-S181THis. The plasmid DNA pKK233-2/BFD-S181T-L476PHis was further digested with *Nhe*I and *Hin*dIII and the 670 bp fragment carrying the mutation C**T**G → C**C**G (L476P) was ligated into the *Nhe*I/*Hin*dIII digested vector pKK233-2/BFDHis lacking the respective fragment yielding the single mutant BFD-L476PHis. The resulting vectors pKK233-2/BFD-S181THis and pKK233-2/BFD-L476PHis were transformed into *E.coli* SG 13009/pREP4 cells.

Saturation mutagenesis

Saturation mutagenesis was performed as described for site-directed mutagenesis with the Quick Change XL kit (Stratagene, La Jolla, CA, USA). The L476 (codon 1426–1428) degenerated saturation mutagenesis primers were: 476satmut1 for (5′GCAGAAAACGTTCCTGGG**WDS**GATG-TGCCAGGGATCG 3′) and 476satmut1 rev (5′CGATCC-CTGGCACATC**SDW**CCCAGGAACGTTTTCTGC 3′) as well as 476satmut2 for (5′GCAGAAAACGTTCCTGGG**S-NS**GATGTGCCAGGGATCG 3′) and 476satmut2 rev (5′CG-ATCCCTGGCACATC**SNS**CCCAGGAACGTTTTCTGC 3′). The plasmid pKK233-2 including the wild-type BFDHis (wtBFDHis) gene was amplified in a PCR reaction (step 1: 95°C, 30 s; step 2: 55°C, 30 s; step 3: 68°C, 15 min; 18 cycles) and the parental DNA template was digested with

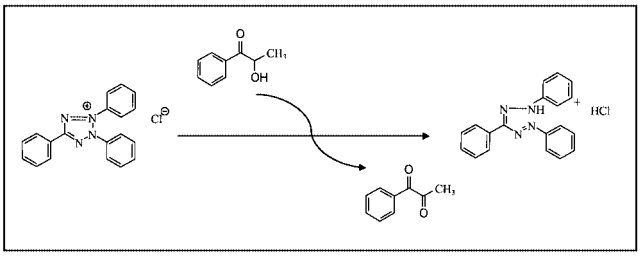

Fig. 2. 2-HPP reduces 2,3,5-triphenyltetrazolium chloride to the respective formazane which has an intense red color.

*Dpn*I. The mutated plasmids (annealed double-stranded nicked DNA molecules) were transformed into XL10-Gold *E.coli* cells, which are able to repair nicks in plasmids (Stratagene) and plated on LB agar plates containing 100 µg/ml ampicillin. Transformed *E.coli* XL 10-Gold cells were pooled, plasmid DNA was prepared and transformed into competent *E.coli* SG 13009/pREP4 cells (Qiagen) which were plated on LB agar plates containing 100 µg/ml ampicillin and 25 µg/ml neomycin. A total of 2×10^3 independent clones were transferred into 96-well plates. Masterplate cultures were grown at 37°C and then stored with 10% glycerol at –80°C.

Selection of variants with BFD activity and stability in organic solvents

For screening, masterplates were replicated into 96-well plates containing 100 µl LB medium with 100 µg/ml ampicillin and 25 µg/ml neomycin and incubated for 20 h at 37°C. A total of 40 µl of each culture was then transferred into 96-deep-well plates containing 1.2 ml LB medium with 100 µg/ml ampicillin and 25 µg/ml neomycin. After incubation at 37°C for 4 h, protein expression was induced by the addition of 1 mM isopropyl-β-D-thiogalactoside (IPTG). The plates were incubated at 37°C for 16 h. Cells were harvested by centrifugation for 15 min at room temperature at 4000 r.p.m. (Rotina 35R Hettich, Tuttlingen, Germany). The cell pellets were dispersed in 100 µl potassium phosphate buffer (50 mM, pH 7.0) containing ThDP (0.5 mM) and $MgSO_4$ (2.5 mM), cell lysis was achieved by the addition of 1 mg/ml lysozyme and incubation at 30°C for 30 min on a shaker. A 50 µl aliquot of each crude extract was then transferred into two fresh 96-well plates, respectively, and 50 µl of 2-fold substrate solution in potassium phosphate buffer (50 mM, pH 7.0) containing 0.5 mM ThDP, 2.5 mM $MgSO_4$, 80 mM benzaldehyde, 1 M acetaldehyde and either 3 or 7.2 M ethanol was added to the crude extract of each clone. The reaction mixture was incubated at 30°C for 4 h. Afterwards, the 96-well plates were centrifuged (15 min, 4000 r.p.m., room temperature) and 100 µl of supernatant was transferred into fresh 96-well plates and assayed for the accumulation of 2-HPP with a colorimetric assay (Breuer *et al.*, manuscript submitted). To each well containing 100 µl supernatant of the reaction mixture 10 µl of 3 M NaOH and then 10 µl of 0.4% 2,3,5,-triphenyltetrazolium chloride (Figure 2) in methanol were added and the formation of the red formazan dye was monitored by measuring the absorbance at 510 nm using a THERMOmax plus plate reader (Molecular Devices, Sunnyvale, CA, USA). The time of the development of the red color was taken as an indicator for the amount of 2-HPP in the mixture. The relation of activities in 1.5 and 3.6 M ethanol was taken as an indicator of activity in the presence of ethanol.

Expression and purification of selected BFDHis variants

Expression and purification of wtBFDHis and BFDHis variants was performed as described for PDC (Pohl *et al.*, 1998) using potassium phosphate buffer (50 mM, pH 7.0) for 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 muteins stored at –20°C were stable for several months.

Protein determination

Determination of protein concentration was performed according to Bradford (Bradford, 1976) using BSA for calibration.

Enzymatic synthesis in buffer and analysis of 2-HPP

To measure initial carboligase activities 10–50 µg purified wtBFDHis or BFDHis mutein were incubated in 0.5 ml of 50 mM KP_i, pH 7.0, containing 0.5 mM ThDP, 2.5 mM $MgSO_4$ in the presence of 20, 40 or 60 mM benzaldehyde and 500 mM acetaldehyde for 30 min at 30°C. After heat inactivation of the enzymes, the resulting 2-HPP formed was measured using an analytical HPLC system (Gynkotek, Germering, Germany) with an ultraviolet monitor (263 nm). Separation was performed on a C18-Hypersil column (C&S, Langerwehe, Germany) using 0.5%/20% acetic acid/acetonitrile (v/v) as eluent. The flow rate was 1.1 ml/min. The retention time for 2-HPP was 12.8 min. The enantiomeric excess (ee) of 2-HPP was investigated by chiral-phase HPLC. The reaction mixtures were extracted with trichloromethane (1:1) and phases were separated by centrifugation (13 000 r.p.m.). Chiral-phase HPLC was performed on a Chiralcel OB (Daicel, Düsseldorf, Germany) using *i*-hexane/isopropanol 95:5 as eluent and a flow rate of 0.75 ml/min.The retention time for (*S*)-2-HPP was 20.6 min and for (*R*)-2-HPP 44.4 min.

Enzymatic synthesis of 2-HPP in the presence of organic solvents

The activity of wtBFDHis and BFDHis muteins in the presence of organic solvents was studied by incubating 20–100 µg/ml purified enzyme in 50 mM KP_i, pH 7.0, containing 0.5 mM ThDP, 2.5 mM $MgSO_4$, 20, 40 or 60 mM benzaldehyde, 500 mM acetaldehyde and 1.5 or 3.6 M ethanol or varying concentrations of DMSO (10–50%) for 30 min at 30°C. After heat inactivation of the enzymes, the 2-HPP formed was measured as described above.

Stability of carboligase activity in the presence of organic solvents

The stability of wtBFDHis and selected BFDHis muteins in the presence of organic solvents was investigated by incubating 50–300 µg/ml purified enzyme in 50 mM KP_i, pH 7.0 with 0.5 mM ThDP, 2.5 mM $MgSO_4$ with or without addition of 1.5 M ethanol or 20% (v/v) DMSO, respectively. The incubations were carried out at 25°C. The carboligase activity of the enzymes was measured over 10 days. At different time intervals aliquots were removed from each incubation mixture and assayed for carboligase activity as described above.

Assay of decarboxylase activity

The decarboxylation of benzoylformate was studied using a coupled enzymatic test as described elsewhere (Iding *et al.*, 2000).

Kinetic measurements were taken at 30°C on a THERMOmax plus microtiterplate reader (Molecular Devices). Initial enzymatic activities were measured in the range of

0.03–60 mM benzoylformate. The assay mixture was prepared from the following stock solutions prepared in potassium phosphate buffer (50 mM, pH 6.0) containing ThDP (0.5 mM) and $MgSO_4$ (2.5 mM): benzoylformate solution (20 µl, 10-fold concentrated ranging from 0.06 to 120 mM, adjusted to pH 6.0), NADH (20 µl, 3.5 mM), horse liver alcohol dehydrogenase (HLADH, 10 µl, 2 U) and potassium phosphate buffer (50 µl, 50 mM, pH 6.0, 0.5 mM ThDP, 2.5 mM $MgSO_4$). The components were mixed in a 96-well microplate, incubated at 30°C, and the reaction was started by the addition of BFD solution (100 µl, 0.025 U). The descending curve was examined at 340 nm and the linear slope was calculated from 0 to 90 s. K_m, V_{max} and K_i values were calculated by a non-linear fitting of the hyperbolic curves according to Michaelis–Menten. One unit is defined as the amount of enzyme that catalyzes the decarboxylation of 1 µmol benzoylformate per minute at pH 6.0 and 30°C.

Stability of cofactor binding

The stability of cofactor binding by the holoenzymes was studied by incubating 0.1 mg/ml wtBFDHis or mutant BFDHis in 50 mM KP_i, pH 6.5 or pH 8.0, without cofactors for 24 h. To follow the time course of inactivation, 50 µl samples were removed and decarboxylation activity was determined with the photometric assay. To measure residual activity, cofactors were omitted from the assay buffer.

Computer graphic studies

The structural models of the different mutations were constructed on the basis of the reported crystal structure of native BFD (Hasson *et al.*, 1998), using the programs WHATIF and Ribbons (Carson, 1991) running on a Silicon Graphics workstation.

Results and discussion

Expression of protein and construction of the BFDHis random mutagenesis library

For the molecular evolution of BFD, the gene from *P.putida* was cloned with a hexahistidine (His) tag into a pKK 233-2 expression vector (Qiagen) that allows high expression levels in the *E.coli* strain SG 13009, the one-step purification of the enzyme via the His tag and the simple cloning of BFD libraries into the vector due to the presence of flanking *Nco*I and *Hin*dIII restriction sites which are not included in the BFD gene. The one-step purification of wtBFDHis yielded protein that is ~95% pure (Iding *et al.*, 2000). The affinity tag does not interfere with the activity of the protein (Iding *et al.*, 2000).

The libraries were constructed by epPCR (Leung *et al.*, 1989; Cadwell and Joyce, 1992; Zhao *et al.*, 1998). The PCR conditions for random mutagenesis were optimized to introduce a limited number of base substitutions into the BFD gene. The effects of several factors decreasing the fidelity of *Taq* DNA polymerase were examined as alteration of the $MgCl_2$ concentration (1.5 or 7 mM), addition of $MnCl_2$ (0 or 0.5 mM) and an imbalance of the deoxynucleotide pool (Table I). The PCR was carried out under four sets of conditions and the PCR products were cloned and transformed into *E.coli* SG13009. A total of 4×10^2 independent clones of each library were tested for carboligation activity. The libraries showed between 4 and 74% inactive clones. Since the mutation frequency should not be too high to enable detection of optimized mutants (Kuchner and Arnold, 1997), the library which showed 53% inactive clones was choosen for screening. Sequencing of several clones from these library revealed one to four nucleotide exchanges in the 1.6 kb BFD gene.

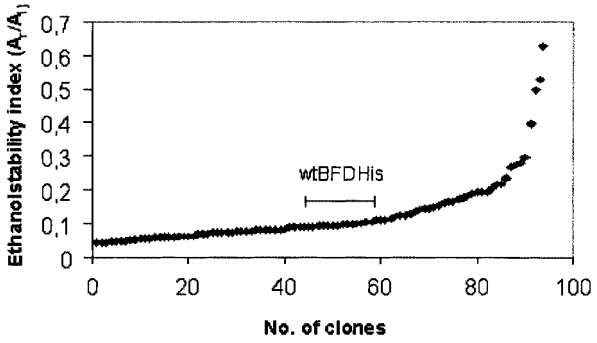

Fig. 3. Transformants of the BFD library were grown in 96-well plates. Initial carboligase activity (A_i) with 1.5 M ethanol in the reaction mixture and residual activity (A_r) with 3.6 M ethanol in the reaction mixture were measured in two separate replica plates using a colorimetric assay. A_r/A_i values of the individual clones are sorted and plotted in ascending order.

Development of a screening system

A total of 4.2×10^3 independent clones from this library were grown in deep-well microtiterplates; protein expression was induced by the addition of IPTG. The cells were harvested by centrifugation and lysis of the cells was carried out by incubation with lysozyme. The crude extracts were incubated with benzaldehyde and acetaldehyde as substrates for carboligation and the formation of 2-hydroxy ketones was monitored by a colorimetric assay using 2,3,5-triphenyltetrazolium chloride (Figure 2), which is reduced by 2-hydroxy ketones leading to a red formazane dye (Breuer *et al.*, manuscript submitted). Tetrazolium salt based reagents are described for detecting acyloins after paper chromatography (Touchstone, 1992). The time of the development of the red color is a measure for the amount of 2-hydroxy ketones, here 2-HPP, formed by the BFD catalysis. The substrates benzaldehyde and acetaldehyde as well as the by-product acetoin do not interfere with this reaction.

Screening of the BFDHis mutant library for mutants with enhanced carboligase activity in water-miscible organic solvents

The random mutagenesis library was screened for carboligase activity in the presence of 1.5 M ethanol using the colorimetric assay. Approximately 50% of the mutant clones from the library showed measurable carboligase activity. Those clones were rescreened for residual carboligase activity in the presence of 3.6 M ethanol. The screening conditions were selected as follows: crude extract of wtBFDHis was incubated with substrate solution containing different concentrations of ethanol. The addition of 1.5 M ethanol was choosen for measuring initial activity (A_i) since wtBFDHis crude extract showed the same activity in the presence of 1.5 M ethanol as in buffer without ethanol. In the presence of 3.6 M ethanol the activity of wtBFD is decreasd to 20%. The relation of residual activity (A_r) and initial activity (A_i) was taken as an indicator for high carboligase activity in the presence of ethanol (Figure 3). Clones with high carboligase activity in buffer and clones with >50% residual activity in the presence of 3.6 M ethanol were examined further.

Characterization of selected mutants

Sequence analysis. Clones 22H6, 22G1, 23C8 and 23E11 (Table IIa) were subjected to DNA sequencing to determine the amino acid substitutions that had been introduced into

Table II. Base pair and amino acid exchanges of mutants isolated from the random mutagenesis library (a) or from the saturation mutagenesis library (b) and comparison of the enzymes carboligase activities (40 mM benzaldehyde, 500 mM acetaldehyde, 30°C) in buffer (50 mM KP_i, pH 7.0, 0.5 mM ThDP, 2.5 mM $MgSO_4$), in the presence of 1.5 M ethanol or 20% (v/v) DMSO with respect to the wild-type BFD

Clone	Base exchange	Amino acid substitution	Carboligase activity (% of wtBFD)		
			Buffer	1.5 M ethanol 20% (v/v)	DMSO
(a)					
22H6	T1427A T1473G	L476Q Silent	395	727	506
22G1	A330G T1427A A1573G C1602A	Silent L476Q S525G Silent	245	373	325
23C8	T514A T1427C	S181T L476P	66	142	175
23E11	T196A T256A	F66I W86R	n.d.	n.d.	n.d.
(b)					
7A12	C1426T T1427C G1428C	L476S	290	420	350
8B12	C1426T T1427G G1428C	L476C	270	515	431
9E12	C1426A	L476M	382	562	423
10F7	C1426A T1427C G1428C	L476T	328	500	398
6A3 10G3	C1426A T1427A	L476K	292	592	500
13H4	C1426G T1427C	L476A	244	558	411
16B2 16B3 20C2	C1426G T1427C G1428C	L476A	n.d.	n.d.	n.d.
13E12 13H2 14B8 16A2 18H12	T1427A	L476Q	n.d.	n.d.	n.d.
18G12	T1427A G1428C	L476H	203	362	319
19E12	C1426G 20C3	L476G T1427G	237	469	427

n.d., not determined.

these clones. They contain between two and four nucleotides exchanges (Table IIa). One position has been mutagenized in three of four clones: Leu 476 to Gln (twice) or Pro (once). The clones 22H6, 22G1 and 23C8 were expressed in *E.coli* SG13009 and purified as described for wtBFDHis. To characterize the effects of the amino acid exchanges of the double mutant BFD-S181T-L476PHis (23C8) the two single mutants corresponding to each of the amino acid subtitutions were constructed, over-expressed and purified by Ni-chelat affinity chromatography.

Characterization of the carboligase activity of the wtBFDHis

The purified wtBFDHis enzyme is sufficiently stable in the presence of water-miscible organic solvents since it retains full activity after 10 days incubation in aqueous EtOH (1.5 M) or DMSO (20%, v/v) at 25°C (data not shown). The stability of wtBFDHis in water-miscible organic solvents at 25°C was examined in buffer, buffer containing 1.5 M ethanol or buffer containing 20% (v/v) DMSO. At different time intervals aliquots were removed from each incubation mixture and assayed for residual carboligation activity under standard conditions.

The initial rate velocities for the synthesis of 2-HPP were determined with 500 mM acetaldehyde and different benzaldehyde concentrations in buffer as well as in the presence of aqueous EtOH or DMSO (Table IIIa). In aqueous buffer benzaldehyde concentrations >40 mM are not soluble. Higher benzaldehyde concentrations lead to a decrease of the initial reaction velocity which might be interpreted in terms of enzyme inactivation (Table IIIa). The K_m value for benzaldehyde was calculated to be higher than the maximal solubility of benzaldehyde in buffer (Dünnwald *et al.*, 2000). This means that the catalytic activity of wtBFDHis is limited by the low solubility of the substrate. In the presence of organic solvents such as 1.5 M EtOH or 20% DMSO 60 mM benzaldehyde can be dissolved resulting in an increased reaction velocity. Compared to the reaction in buffer, 1.5 M EtOH decreased the activity to ~40%, whereas DMSO had no significant effect on the carboligase activity of the purified wtBFDHis.

Carboligase activity of selected BFDHis variants

We examined the carboligase activity of the purified BFDHis muteins 22G1, 22H6, 23C8 yielded by random mutagenesis as well as of the single mutants BFD-S181THis and BFD-L476PHis cloned from the double mutant 23C8. The initial rate velocities for the synthesis of 2-hydroxypropiophenone in buffer and in the presence of organic solvents were determined with 500 mM acetaldehyde and different concentrations of benzaldehyde (Table IIIb).

Both L476Q mutants (22G1 and 22H6; Table II) showed a ~4-fold higher carboligase activity in aqueous buffer compared to the wild-type enzyme (Table IIIb) with a slight decrease in activity in the presence of 60 mM benzaldehyde. Addition of 1.5 M ethanol to the L476Q variants resulted in a 30–40% decrease of activity compared to their activity in buffer. In contrast, in the presence of 20% (v/v) DMSO the activity of the L476Q variants increased slightly compared to their activity in buffer. In the presence of DMSO the carboligase activity increased with increasing benzaldehyde concentrations up to 60 mM.

Both L476P mutants (single mutant L476P and 23C8; Table II) revealed lower carboligase activity than the wtBFDHis in buffer, whereas these variants gained 80% of their activity in the presence of 1.5 M ethanol and showed a prominent 2–3-fold increase of carboligase activity in the presence of 20% (v/v) DMSO with an additional increase of activity at 60 mM benzaldehyde (Table IIIb). The single mutant S181T showed the same activity like the wtBFDHis in aqueous buffer as well as in the presence of 1.5 M ethanol or 20% (v/v) DMSO (Table IIIb) indicating that the L476 exchange was responsible for the effects on the carboligase activity of the variants derived by random mutagenesis.

Like wtBFDHis, all enzymes retain full carboligase activity after 10 days incubation in buffer containing 1.5 M ethanol or 20% (v/v) DMSO (data not shown). Mutant BFD-L476QHis was investigated for the pH and temperature optima of the carboligase reaction. The pH optimum for the carboligation of benzaldehyde and acetaldehyde to yield (*S*)-2-HPP was determined to pH ⩾8.0 (data not shown) as for the wild-type BFD (Iding *et al.*, 2000). The highest carboligase activity of both enzymes, the wtBFDHis and the L476Q variant, was

Table III. Carboligase activity of wtBFDHis (a) and mutants derived by random mutagenesis (b) as well as by saturation mutagenesis (c)

Enzyme (U/mg)	Buffer			1.5 M ethanol			20% (v/v) DMSO		
Benzaldehyde (mM)	20	40	60	20	40	60	20	40	60
(a)									
wtBFDHis	6.2 ± 0.6	7.1 ± 0.7	7.0 ± 0.7	2.3 ± 0.3	2.6 ± 0.4	3.2 ± 0.4	4.8 ± 0.2	6.4 ± 0.7	7.1 ± 0.7
(b)									
S181T	6.6 ± 0.6	7.5 ± 0.8	7.4 ± 0.7	2.5 ± 0.2	2.4 ± 0.4	2.9 ± 0.3	5.5 ± 0.4	7.2 ± 0.7	7.9 ± 0.8
S181T-L476P	4.8 ± 0.4	4.7 ± 0.4	3.7 ± 0.2	4.1 ± 0.3	3.7 ± 0.4	3.7 ± 0.2	8.4 ± 0.3	11.2 ± 0.5	11.1 ± 1.1
L476P	4.8 ± 0.4	4.6 ± 0.2	3.8 ± 0.1	3.6 ± 0.1	3.7 ± 0.2	3.3 ± 0.1	8.6 ± 0.3	10.7 ± 0.5	11.9 ± 0.6
L476Q-S525G	16.1 ± 1.2	17.4 ± 1.3	15.7 ± 0.5	9.2 ± 1.1	9.7 ± 1.0	11.8 ± 1.4	15.7 ± 0.6	20.8 ± 2.0	22.6 ± 1.9
L476Q	25.6 ± 3.3	28.1 ± 2.5	27.0 ± 1.2	15.8 ± 1.6	18.9 ± 1.1	20.4 ± 0.9	26.8 ± 3.4	32.4 ± 1.7	36.4 ± 2.4
(c)									
L476S	17.1 ± 0.2	20.6 ± 0.6	19.8 ± 0.5	10.2 ± 0.2	11.7 ± 1.0	11.8 ± 0.2	18.0 ± 0.4	22.4 ± 1.3	26.2 ± 1.0
L476C	16.9 ± 1.1	19.2 ± 1.0	17.6 ± 0.3	11.7 ± 1.0	13.4 ± 1.8	13.2 ± 1.0	20.8 ± 1.7	27.6 ± 2.1	29.6 ± 1.0
L476M	23.8 ± 0.9	27.1 ± 1.4	28.2 ± 2.8	13.3 ± 1.4	14.6 ± 2.0	16.7 ± 1.9	21.5 ± 0.3	27.1 ± 1.2	29.4 ± 1.0
L476T	18.7 ± 2.1	23.3 ± 1.2	22.6 ± 2.1	11.4 ± 1.5	13.0 ± 2.2	14.5 ± 1.7	19.5 ± 1.7	25.5 ± 1.2	29.4 ± 1.0
L476A	16.1 ± 0.8	17.3 ± 1.5	16.8 ± 1.9	12.7 ± 0.6	14.5 ± 1.6	15.3 ± 0.3	20.0 ± 1.7	26.3 ± 2.5	29.0 ± 2.0
L476G	14.6 ± 1.6	16.8 ± 1.7	15.0 ± 0.5	11.0 ± 1.2	12.2 ± 0.9	11.6 ± 1.6	19.7 ± 2.5	27.3 ± 2.8	27.4 ± 2.3
L476H	13.7 ± 1.1	14.4 ± 0.8	12.9 ± 2.7	9.2 ± 2.3	9.4 ± 2.8	8.6 ± 1.1	16.8 ± 3.2	20.4 ± 5.2	21.0 ± 4.6
L476K	18.2 ± 0.4	20.7 ± 0.2	17.6 ± 1.2	14.6 ± 0.8	15.4 ± 1.6	15.6 ± 2.8	24.4 ± 1.0	32.0 ± 0.4	33.4 ± 0.7

Initial rate velocities were meassured with purified enzymes in buffer, in the presence of 1.5 M ethanol or 20% (v/v) DMSO with different concentrations of benzaldehyde and 500 mM acetaldehyde. All values (U/mg) are averages based on values from at least independent triplicate assays.

observed at 40°C, but the wild-type enzyme revealed a higher residual activity at 60°C reaction temperature compared to the L476 Q variant (data not shown).

Saturation mutagenesis at position L476

Due to the low mutation rate used during generation of the BFD mutant library not all of the remaining 19 amino acids could be introduced at each position because introduction of certain amino acids would require two or three base substitutions per codon. As a sensitive position in the protein that is important for improving the enzymes side reaction has been identified, saturation mutagenesis was applied to position L476 to introduce different amino acids. This method was shown to be a valuable completion to directed evolution (Miyazaki and Arnold, 1999). Site-directed mutagenesis with two sets of degenerative PCR primers was carried out yielding a library of 2000 independent clones which were screened for carboligase activity. More than 50% of the library showed very high carboligase activity, so that crude extracts had to be diluted with buffer in order to measure in the linear range of the colorimetric assay, indicating that this position is quite important for carboligase activity. Assuming that all 19 amino acid substitutions had been generated by the saturation mutagenesis the library was assayed for activity in the presence of 1.5 and 3.6 M ethanol. Nineteen clones showing carboligase activity well above the wild-type level were further characterized. DNA sequencing of these clones revealed nine different mutations (Table IIb), some were found several times. Except for the L476M substitution, multiple base exchanges were required for these replacements. Seven of the nine new muteins resulted in non-conservative substitutions.

Role of BFD position 476 in carboligase activity

The nine different L476 variants obtained by saturation mutagenesis and screened for high carboligase activity were purified and characterized. The expression levels of the BFD variants were the same as for the wild-type enzyme with ~10–15% of recombinant enzyme among the total soluble cell protein. All variants showed significantly higher carboligase activity compared to wtBFDHis in buffer as well as in the presence of water-miscible organic solvents (Table IIIc). For most variants the reaction velocity in the presence of organic solvents was found to be proportional to the benzaldehyde concentration applied due to the increased solubility and distribution of the substrate. The tested organic solvents did not only influence the solubility of the substrate benzaldehyde but showed intrinsic effects on the enzymes. The addition of 1.5 M ethanol slightly decreased the carboligase activity of the BFD variants, but all L476 mutants showed higher residual activity in the presence of 1.5 M ethanol than the wtBFDHis or variant S181T (Table IIIa and b). This means that the stability of the variants towards inactivation by ethanol is enhanced compared to the wildtype. Interestingly, all variants are more active in the presence of 20% (v/v) DMSO compared to their activity in buffer at a given benzaldehyde concentration. This was found especially for variants with hydrophobic (P, A, G) or basic (K, H) residues at position 476. Inactivation as well as stimulation of enzyme activity at low concentrations of organic solvents is described in the literature (Singh and Wang, 1979; Guagliardi *et al.,* 1989; Kudryashova *et al.*, 1997; Gladilin and Levashov, 1998; Blackwood and Bucke, 2000), but very little is known about the effects of such solvents on the catalytic and structural properties of these enzymes.

Recently, a variant of subtilisin E from *Bacillus subtilis* was created to be more active in organic solvents such as DMF (Takagi *et al.*, 2000). In this subtilisin mutant two charged surface residues were substituted by uncharged Cys residues. You and Arnold (You and Arnold, 1994) applied directed evolution on subtilisin E from *B.subtilis* to enhance total activity in aqueous DMSO which resulted in a variant with 157-fold enhanced activity over the parent in 60% (v/v) DMSO.

Stability of BFD variants in the presence of organic solvents

Like the BFD wild-type the BFD variants derived from saturation mutagenesis were investigated for stability in aqueous buffer, buffer containing 1.5 M ethanol or 20% (v/v) DMSO (data not shown). After 10 days incubation all variants retained full activity, demonstrating that the mutations that enhance catalytic activity have no significant effect on the

Table IV. Enantioselectivity of wtBFDHis (a) and mutants derived by random mutagenesis (b) as well as by saturation mutagenesis (c)

Enzyme (S)-2-HPP (% ee)	Buffer			1.5 M ethanol	20% (v/v) DMSO
Benzaldehyde (mM)	20	40	60	40	40
(a)					
wtBFDHis	85	83	81	82	82
(b)					
S181T	n.d.	86	n.d.	84	86
S181T-L476P	n.d.	92	n.d.	92	92
L476P	94	94	92	95	93
L476Q-S525G	n.d.	94	n.d.	n.d.	n.d.
L476Q	95	95	96	95	93
(c)					
L476S	94	92	92	93	94
L476C	95	95	94	95	95
L476M	94	93	90	93	94
L476T	94	94	92	93	94
L476A	96	96	95	96	96
L476G	94	95	93	94	95
L476H	93	93	93	92	93
L476K	97	96	96	96	96

Influences on the ee of (*S*)-2-HPP were investigated in the presence of 1.5 M ethanol or 20% (v/v) DMSO with different concentrations of benzaldehyde and 500 mM acetaldehyde.
n.d., not determined

enzyme stability. You and Arnold (You and Arnold, 1994) obtained similar results for subtilisin E from *B.subtilis*. Mutations enhancing the catalytic activity occur without changes in stability indicating that both properties are not coupled.

Enantioselectivity of the carboligation reaction

The enantioselectivity of wtBFDHis and variants concerning the synthesis of (*S*)-2-HPP and the influence of the water-miscible organic solvents on the enantioselectivity for the reaction was investigated. In a previous paper we reported that for the wtBFDHis the enantioselectivity of (*S*)-2-HPP formation was found to be a function of temperature and benzaldehyde concentration (Iding *et al.*, 2000) showing decreasing ee of (*S*)-2-HPP with increasing temperature and benzaldehyde concentration. Under the given reaction conditions (500 mM acetaldehyde, 20, 40 or 60 mM benzaldehyde, 30°C reaction temperature) wtBFDHis showed slightly decreasing enantioselectivity with increasing benzaldehyde concentrations ranging from 85 to 81% ee (Table IVa). All BFD variants with an amino acid exchange at position L476 revealed significantly higher enantioselectivity (Table IVb and c) compared to the wtBFDHis with ee values from 92 to 97%. For most of the BFD variants a slight decrease in enantioselectivity with increasing benzaldehyde concentration was demonstrated as well. Only variant S181T showed ee values in the range of the wild-type enzyme with 86–84%, strongly indicating that the exchange at position L476 is responsible for the improved enantioselectivity. A recent publication describes similar results from a lipase from *Pseudomonas aeruginosa*, where the flexibility of certain loop structures which are located far from the stereocenter of the substrate influence enantioselectivity (Liebeton *et al.*, 2000). Addition of either ethanol or DMSO to the reaction mixture did not influence the enantioselectivity of all BFDHis variants as well as the wtBFDHis (Table IVa–c).

Thus, with all L476 muteins an improvement of enantioselectivity under reaction conditions that are useful in technical applications, e.g. 30°C reaction temperature or high benzaldehyde concentration, could be achieved, although enantioselectivity was not a selection parameter for screening of the random mutagenesis library.

Role of position 476 in the decarboxylation reaction

The decarboxylation of aromatic 2-keto acids is the main reaction of BFD which was not monitored during the screening of the libraries. To test the influence of the mutation of position L476 on the main reaction of BFD, the decarboxylation of benzoylformate, was investigated with the muteins derived from random and saturation mutagenesis (Table V). Decarboxylase activity of muteins with exchanges in position 476 decreases in the series: S > K > L (wild type) > T > Q > H > M > C > A > G > P. This is not associated with significant changes of the K_m value for the substrate benzoylformate. The decarboxylase activity of the wtBFDHis is inhibited by high concentrations of the substrate benzoylformate with a K_i value of ~143 mM. This effect was enhanced by most of the mutations up to a K_i value of 45 mM for the mutein L476P.

Investigation of the ratio of decarboxylase versus carboligation activity revealed a preference of the carboligase side reaction over the main reaction for all variants (Table V). In a very recent publication on the closely related pyruvate decarboxylase from yeast, Sergienko and Jordan (Sergienko and Jordan, 2001) described active center mutations which led to an two to three orders of magnitude lower catalytic efficiency of the decarboxylase reaction combined with a higher rate of the carboligase reaction.

Stability of cofactor binding

For ThDP- and Mg^{2+}-dependent decarboxylases the addition of the cofactors to the reaction buffer is necessary because the cofactors are non-covalently bound (Pohl, 1997). We studied the cofactor binding in the wtBFDHis and the mutant enzymes by incubating the holoenzymes in cofactor-free buffer and determined the time course of inactivation by measuring the residual decarboxylation activity over 24 h (Table V). wtBFDHis and S181T showed no loss of activity in cofactor-free buffer over 24 h. Variants L476M, L476S, L476Q and L476T showed a decrease of activity of ~40% over 24 h, whereas variants L476A, L476G, L476C, L476P, L476K and L476H are rapidly inactivated in cofactor-free buffer with a $t_{1/2}$ lower than 10 min (Table V), indicating that the residue at position 476 influenced binding of the cofactors significantly. Decarboxylase activity of all variants could be restored by the addition of ThDP and magnesium ions to the reaction mixture (data not shown).

Computer graphic studies

Based on the crystal structure of BFD (Hasson *et al.*, 1998) the structural position of Leu476 was investigated. The quaternary structure of the enzyme is a homotetramer. Each monomer consists of three domains α, β and γ. The cofactor ThDP is bound at a dimer interface with the binding site formed by the α-domain of one monomer and the γ-domain of another. The BFD tetramer can be described as a dimer of dimers with four active sites. Figure 4 gives a partial view on the active site at the interface between two monomers. The analysis showed that Leu476 is part of a loop on the surface of one monomer located near the active center cavity. The start and endpoint of the loop are two glycine residues (456 and 480),

Table V. Activity and stability of cofactor binding of the decarboxylation reactions and ratio of decarboxylation versus carboligation activity (50 mM KP_i, pH 7.0, 0.5 mM ThDP, 2.5 mM $MgSO_4$, 40 mM benzaldehyde, 500 mM acetaldehyde, 30°C) of wtBFDHis (a) and mutants isolated from the random mutagenesis library (b) as well as mutants generated by saturation mutagenesis (c)

Enzyme	$K_{m\ (benzoylformate)}$ (mM)	$K_{i\ (benzoylformate)}$ (mM)	V_{max} (U/mg)	$t_{1/2}$ in cofactor free buffer (min)	Decarboxylation/ carboligation
(a)					
wtBFDHis	0.54 ± 0.09	143 ± 38	327 ± 12	>1440	46.0
(b)					
S181T	1.23 ± 0.04	99 ± 0.2	236 ± 4	>1440	31.5
S181T-L476P	0.6 ± 0.05	78 ± 9	150 ± 14	n.a.	31.9
L476P	1.03 ± 0.04	45 ± 2	63 ± 1	n.a.	13.7
L476Q-S525G	1.06 ± 0.07	56 ± 1	233 ± 14	n.d.	13.4
L476Q	0.58 ± 0.07	100 ± 27	246 ± 16	150	8.8
(c)					
L476S	1.14 ± 0.08	62 ± 5	359 ± 7.9	>1440	17.4
L476C	1.26 ± 0.07	49 ± 3	166 ± 3.2	<10	8.6
L476M	1.08 ± 0.09	104 ± 12.5	213 ± 5.2	>1440	7.9
L476T	1.02 ± 0.06	60 ± 4.6	318 ± 6.5	60	13.6
L476A	1.17 ± 0.08	54 ± 4.1	141 ± 6.3	<10	8.2
L476G	0.59 ± 0.07	130 ± 23.9	90 ± 5.1	<10	5.4
L476H	1.0 ± 0.05	67 ± 3.7	233 ± 3.3	<10	16.2
L476K	1.46 ± 0.11	62 ± 5.8	346 ± 4.6	<10	16.7

n.a., no activity meassured; n.d., not determined ($n = 6$).

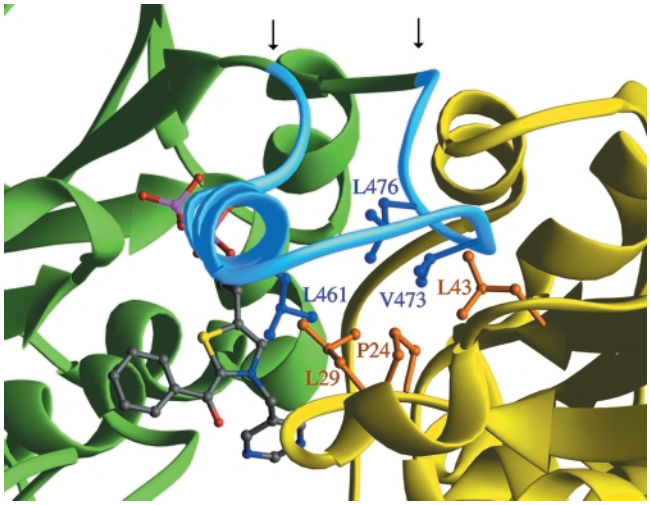

Fig. 4. Partial view into the 3D structure of wtBFD showing one active center at the interface of two monomers (green and yellow). L476 is located in a loop (blue, belonging to the green monomer) on the surface of the protein and is involved in a hydrophobic network anchoring the loop on the other monomer (yellow). The substrate benzaldehyde bound to the cofactor ThDP is shown in gray. Arrows indicate the positions of the two glycine residues marking the start and endpoint of the loop.

which may function as hinges. This loop covers the active center like a lid forming an hydrophobic cavity (Figure 4). Residues Gly459 to Gly466 form an α-helix which is directly involved in cofactor binding (Hasson *et al.*, 1998). Residues Val467 to Phe483 are involved in contacts between monomers in the dimer. Residues Val473 and Leu476 of one monomer and Lys32 of the other monomer build a hydrophobic network. Polar or basic residues at position 476 could weaken the hydrophobic interaction between the two monomers. Thus, a faster entry of substrates in or a faster exit of product out of the active center could be possible because the lid is not locked firmly. This could also explain the rapid loss of cofactors of most L476 mutants (Table V). Dordick (Dordick, 1992) mentioned one approach to increase the flexibility of the active site by increasing active site polarity. This is confirmed by a study with subtilisin BNP′ (Dordick, 1992) indicating that increased polarity in the active site of the enzyme could improve catalytic activity in organic solvents with and without the addition of water. Exchange of Leu476 by other hydrophobic residues could maintain the hydrophobic network.

A direct interaction of L476 and L461 is possible, which could be influenced by the exchange at position L476. The residue L461 could probably support the V conformation of the cofactor ThDP which is energetically unfavorable, but is common among the ThDP-dependent enzymes. Guo *et al.* (Guo *et al.*, 1998) showed that a hydrophobic amino acid is required to maintain the reactive V conformation of ThDP-dependent enzymes. In yeast pyruvate decarboxylase (PDC) which is closely related to BFD, I415 was found to be responsible for supporting the V coenzyme conformation. Although L461 in BFD is not the corresponding residue to I415 in PDC it is involved in binding of the cofactor ThDP, but not in binding the pyrimidine ring as in PDC (Arjunan *et al.*, 1996) but in binding a phosphate group (Hasson *et al.*, 1998). The increased carboligase activity could be modulated by a greater coenzyme flexibility which would be evidenced by a looser binding of ThDP. Although the residue at position 476 is not located in the direct vicinity of the active center, it could also be possible that just conformational changes of residues involved in the catalytic reaction could accelerate the reaction and could weaken the binding of the cofactors. A subtilisin variant from *B.subtilis* that showed enhanced activity in aqueous DMSO solutions was created by a directed evolution approach (You and Arnold, 1994). This subtilisin variant contained 10 amino acid substitutions that were clustered on the face of the enzyme that harbors the active site and substrate binding pocket and were located in loop structures connecting elements of secondary structure.

Conclusions

BFD is a useful catalyst for organic synthesis, particularly for the enantioselective formation of α-hydroxy ketones. The productivity of the enzyme is limited by the solubility of the aromatic aldehyde substrates in aqueous buffers. With a combination of random and saturation mutagenesis we suc-

ceeded in enhancing the carboligase activity of BFD, which is a side reaction of the enzyme of hitherto unknown physiological function. Random mutagenesis showed that L476 plays an important role in the carboligase activity of BFD. Saturation mutagenesis at this position resulted in 10 variants with up to 5-fold enhanced carboligase activity, which were activated by a low concentration of DMSO. The improvement of activity had no deleterious effect on the enantioselectivity of the variants, since the L476 variants showed higher enantioselectivity than the wild type under conditions that are useful for synthetic application. There is excellent potential for improving a side reaction of an enzyme because it is not optimized by nature itself. As shown by Chen *et al.* (Chen *et al.*, 1991), additive effects of single amino acid substitutions that increase activity and stability of an enzyme will lead to further improvements in the future.

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