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Changing the Substrate Reactivity of 2-Hydroxybiphenyl 3-Monooxygenase from *Pseudomonas azelaica* HBP1 by Directed Evolution*

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The substrate reactivity of the flavoenzyme 2-hydroxybiphenyl 3-monooxygenase (EC 1.14.13.44, HbpA) was changed by directed evolution using error-prone PCR. *In situ* screening of mutant libraries resulted in the identification of proteins with increased activity towards 2-*tert*-butylphenol and guaiacol (2-methoxyphenol). One enzyme variant contained amino acid substitutions V368A/L417F, which were inserted by two rounds of mutagenesis. The double replacement improved the efficiency of substrate hydroxylation by reducing the uncoupled oxidation of NADH. With guaiacol as substrate, the two substitutions increased V_{max} from 0.22 to 0.43 units mg^{-1} protein and decreased the K'_m from 588 to 143 μM, improving k'_{cat}/K'_m by a factor of 8.2. With 2-*tert*-butylphenol as the substrate, k'_{cat} was increased more than 5-fold. Another selected enzyme variant contained amino acid substitution I244V and had a 30% higher specific activity with 2-*sec*-butylphenol, guaiacol, and the "natural" substrate 2-hydroxybiphenyl. The K'_m for guaiacol decreased with this mutant, but the K'_m for 2-hydroxybiphenyl increased. The primary structure of HbpA shares 20.1% sequence identity with phenol 2-monooxygenase from *Trichosporon cutaneum*. Structure homology modeling with this three-domain enzyme suggests that Ile^{244} of HbpA is located in the substrate binding pocket and is involved in accommodating the phenyl substituent of the phenol. In contrast, Val^{368} and Leu^{417} are not close to the active site and would not have been obvious candidates for modification by rational design.

2-Hydroxybiphenyl 3-monooxygenase (EC 1.14.13.44; HbpA) belongs to the family of flavoprotein hydroxylases (1–3). These enzymes are involved in many important biological processes, such as the biosynthesis of cholesterol or the degradation of xenobiotics in mammals and in nature (4–6).

HbpA was first found in *Pseudomonas azelaica* HBP1, a soil bacterium that is able to grow on the fungicide 2-hydroxybiphenyl as sole source of carbon and energy (7). HbpA catalyzes the *ortho*-hydroxylation of 2-hydroxybiphenyl to 2,3-dihydroxybiphenyl, which is then converted to 2-hydroxy-6-phenyl-6-oxo-2,4-hexadienoic acid by a *meta* ring cleavage dioxygenase (HbpC). 2-Hydroxy-6-phenyl-6-oxo-2,4-hexadienoic acid is hydrolyzed by HbpD to benzoate and 2-hydroxy-2,4-pentadienoic acid (7, 8), which are further metabolized via intermediates also formed in the analogous biphenyl degradation pathway (9, 10). HbpA has a broad substrate spectrum, catalyzing the regioselective *ortho*-hydroxylation of a wide range of 2-substituted phenols to the corresponding catechols (Fig. 1) (7, 11). Recently, the *hbpA* gene was cloned into *Escherichia coli*, and this recombinant biocatalyst has been used for the production of different 3-substituted catechols (12, 13). One of these, 3-phenylcatechol, was produced on a kilogram scale, showing that the biocatalytic production of 3-substituted catechols is a possible alternative to chemical synthesis routes (14).

HbpA mutants with an altered substrate reactivity should allow the synthesis of catechols that are not synthesized by wild-type HbpA. Rational protein design based on a known three-dimensional structure has been used for such purposes (15–18), but random approaches have lately become more popular (19). Directed enzyme evolution (20–23), the most often used strategy, was applied to improve substrate specificity, activity, enantioselectivity, or thermostability (21, 24–27).

Here we report on the use of directed evolution to change the substrate reactivity of HbpA. We increased the specific activity of HbpA towards 2-hydroxybiphenyl, 2-*sec*-butylphenol, guaiacol (2-methoxyphenol), and 2-*tert*-butylphenol. Moreover, a significant increase in the efficiency of NADH utilization was achieved with one mutant monooxygenase. These results are interpreted at the structural level with the help of a three-dimensional model of HbpA.

MATERIALS AND METHODS

Chemicals, Bacterial Strains, and Plasmids—*E. coli* JM101 and plasmid pAA1 were used for cloning and gene expression. Plasmid pAA1 is a pUC18 (28) derivative harboring the *hbpA* gene as a *Sal*I/*Nsi*I fragment (29) cloned into the *Sal*I/*Pst*I sites of the pUC18 polylinker.

Commercially available chemicals were purchased from Fluka AG (Buchs, Switzerland). Catalase from beef liver was obtained from Roche Molecular Biochemicals. *Taq* DNA polymerase, restriction enzymes, and T4 DNA ligase were purchased from Roche Molecular Biochemicals. 2,3-Dihydroxybiphenyl and 3-*sec*-butylcatechol were prepared by whole-cell biotransformations, using a recombinant *E. coli* JM101 containing the *hbpA* gene (14).

Random Mutagenesis—The *hbpA* gene (1758 bp) in pAA1 was amplified using *in vitro* manganese mutagenesis (30). For the PCR, the M13/pUC-40 primers (MWG-Biotech GmbH, Münchenstein, Switzerland) were used, each of which complements a 23-bp region of the

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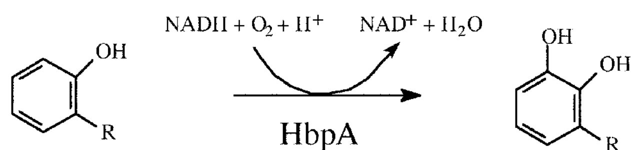

R = phenyl, 2-OH-phenyl, methyl, ethyl, propyl, *i*-propyl, butyl, *sec*-butyl, fluoro, chloro, bromo, or iodo.

FIG. 1. **Reaction catalyzed by wild-type HbpA.** The substrate spectrum of wild-type HbpA is shown.

cloning vector. A 100-μl volume containing 50 mM KCl, 10 mM Tris-HCl (pH 9), 6.5 mM $MgCl_2$, 0.1% Triton X-100, 10 μl of Me_2SO, 0.5 mM $MnCl_2$, 1 mM dNTPs, 15 pmol of each primer, 20 ng of template DNA, and 2.5 units of *Taq* DNA polymerase (Promega, Madison, WI) was placed in a PerkinElmer Life Sciences thermal cycler well. After 5 min at 95 °C, the thermal cycler performed 25 cycles of the following steps: 1 min at 95 °C, 1 min at 55 °C, 2 min at 72 °C. Prior to restriction, the amplified DNA was purified with a DNA clean-up kit (Genomed GmbH, Bad Oeyenhausen, Germany, or Macherey-Nagel AG, Oensingen, Switzerland).

Cloning Procedures—Competent *E. coli* JM101 cells were prepared using a modified $CaCl_2$-based method (31). A 5-ml culture of *E. coli* JM101 was grown overnight in LB. The cells were diluted 1:100 into fresh LB and grown until they reached an A_{600} of 0.5 ± 0.1. After centrifugation (5500 × *g*, 4 °C, 8 min), the supernatant was discarded, and the pellet was resuspended in 0.2 volumes of 10 mM sodium acetate (pH 5.8), 50 mM $MnCl_2$, and 5 mM NaCl. The suspension was put on ice for 30 min and recentrifuged as before. The pellet was dissolved in 0.1 volumes of 10 mM sodium acetate (pH 5.8), 70 mM $CaCl_2$, 5 mM $MnCl_2$, and 5% glycerol and stored in aliquots of 100 μl at −70 °C until use.

Ligation mixtures containing pUC18 (cut *Bam*HI/*Sph*I), amplified *hbpA* gene (cut *Bam*HI/*Sph*I), 0.5 units of T4 DNA ligase (Roche Molecular Biochemicals), and 10× ligation buffer were incubated overnight at 4 °C. The ligation mixture was directly used for transformation. For this, an aliquot of competent *E. coli* JM101 was thawed on ice. The cells were mixed with 1 μl of ligation mixture and placed on ice for 30 min. The heat pulse was performed at 42 °C for 90 s and followed by incubation on ice for 1 min. After the addition of 1 ml of LB, the cells were incubated at 37 °C for 1 h. The complete transformation mixture was transferred onto selective LB plates and incubated overnight at 30 °C.

Screening—The screening procedure for the desired modified HbpA was based on the instability of the reaction products. At neutral pH, catechols autoxidize to quinones and semiquinones, which readily form reddish or brownish, undefined, high molecular weight compounds (32). After transformation, *E. coli* JM101 transformants were transferred directly onto LB plates containing 150 μg ml^{-1} ampicillin, 0.04‰ (w/v) 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal), 200 μM isopropyl-1-thio-β-D-galactopyranoside, and a 0.1–0.5 mM concentration of the 2-substituted phenol to be screened for. Incubation at 30 °C resulted in three colony types: blue colonies, which did not contain the amplified *hbpA* gene; white colonies, which contained an *hbpA* gene that encoded for an inactive enzyme towards the aromatic test substrate, or an inactive *lacZ* gene due to frameshifted ligation; and reddish brown colonies, which contained an enzyme with activity towards the added 2-substituted phenol. The time-dependent intensity of color formation in the latter colonies could be used to distinguish between different activities. For substrates that were only poorly transformed to the corresponding catechol, the color formation could be intensified by the addition of 1.5 mM ferric chloride and 50 μg ml^{-1} *p*-toluidine (33). If positive clones were detected, the plasmids containing the amplified *hbpA* genes were isolated and retransformed into *E. coli* JM101.

Preparation of Cell Extracts—Cells from a 5-ml LB culture were spun down at 5000 × *g* for 15 min and resuspended in 800 μl of 50 mM phosphate buffer (pH 7.2). This suspension was transferred to a 1.5-ml Eppendorf tube containing 1.2-g glass beads (diameter, 0.1–0.2 mm), and the cells were disrupted in a Retsch mill (Retsch GmbH, Hann, Germany) for 10 min at 90% power. The cell extracts were separated from the glass beads by centrifugation (15,000 × *g*, 15 min) and supplemented with FAD to a final concentration of 50 μM. Cell extracts could be stored at −20 °C for 2–3 weeks without significant loss of HbpA activity.

Enzyme Purification—The purification of HbpA and its mutants was based on a simplified version of a procedure described earlier (1). 6 g (cell wet weight) of frozen *E. coli* JM101, harboring a pUC18 derivative encoding either the wild-type or the amplified *hbpA* gene, were suspended in 25 ml of phosphate buffer (10 mM, pH 7.5). Cell extract was prepared by twice passing the suspension through a French pressure cell (20 K; Sim Aminco) at 70 bars, followed by ultracentrifugation at 4 °C (Beckmann L8–60 M, 40,000 × *g*, 30 min).

The clarified cell extract was diluted 1:1 with triethanolamine-HCl buffer (10 mM, pH 7.5) and loaded directly onto an anion exchange column (1.5 × 15 cm; Fractogel EMD DMAE-650 (S); Merck) equilibrated with 10 mM triethanolamine-HCl buffer (pH 7.5). Elution was carried out with a linear gradient from 0 to 1 M NaCl in starting buffer.

Fractions containing HbpA activity were pooled, supplemented with 0.9 M ammonium sulfate, and loaded onto a hydrophobic interaction chromatography column (1 × 8 cm; butyl-Sepharose 4 Fast Flow; Amersham Biosciences, Inc.) equilibrated with 0.75 M ammonium sulfate in 100 mM sodium phosphate buffer (pH 7.0). Elution was carried out with a linear gradient from 0.75 to 0 M ammonium sulfate in 100 mM sodium phosphate buffer (pH 7.0). Fractions containing HbpA were pooled and concentrated in an Ultrafree-15 centrifugal filter device (Biomax-50K; Millipore Corp., Bedford, MA).

Concentrated enzyme (0.3 ml) was supplemented with 0.3 mM FAD and passed through a Superdex 200 gel filtration column (1.6 × 60 cm; Amersham Biosciences) equilibrated with 50 mM sodium phosphate buffer (pH 7.5). Enzyme purity was assessed with SDS-PAGE (12% polyacrylamide), followed by staining with Coomassie Brilliant Blue.

Analytical Methods—As is also the case for other flavin-containing oxygenases, NADH oxidation is partially uncoupled from substrate hydroxylation in HbpA (1). Therefore, specific activities were determined both for NADH oxidation and substrate consumption/product formation. NADH oxidation was followed spectrophotometrically at 340 nm or polarographically by monitoring oxygen consumption with an oxygen electrode (7). The assay contained 0.2–1 mM substrate, 0.3 mM NADH, 20 mM air-saturated phosphate buffer (pH 7.5), and 10–20 μl of cell extract or purified protein in a total volume of 1 ml. To determine substrate utilization and product formation, the enzymatic reaction was stopped by the addition of perchloric acid, and the resulting precipitate was removed by centrifugation (15,000 × *g*, 10 min). The samples were diluted 1:1 with MeOH, 0.1% phosphoric acid and analyzed with a Hypersil ODS column (5 μm, 4.5 × 125 mm) using a Hewlett Packard HP 1050 Ti high pressure liquid chromatograph coupled to a diode array detector (HP DAD 1040M). The elution was carried out under isocratic conditions with MeOH/H_2O (0.1% phosphoric acid) as mobile phase.

Steady-state kinetic parameters were calculated by weighted nonlinear regression analysis (Enzfitter; Elsevier-Biosoft, UK). Uncoupling of product formation from oxygen consumption was resolved by performing HbpA activity assays in the absence and in the presence of catalase (34). The catalase recycles 50% of the oxygen, which is used for hydrogen peroxide formation. By determining initial reaction rates in the absence and presence of catalase, the substrate related uncoupling of product formation from oxygen consumption could be calculated.

HbpA Modeling—The sequence of HbpA was aligned to the recently corrected sequence of phenol 2-monooxygenase from *Trichosporon cutaneum* (35) with ClustalX (36) using the PAM 350 matrix. Model building of HbpA was performed with MODELLER (37) using the CVFF force field (38). The closed form of the phenol 2-monooxygenase structure (Protein Data Bank entry 1foh) was used as a template. The model was verified after several rounds of energy minimization. The stereochemical quality of the homology model was verified by PROCHECK (39), and the protein folding was assessed with PROSAII (40), which evaluates the compatibility of each individual residue with its environment. The FAD and the substrate 2-hydroxybiphenyl were placed in identical positions and orientations as the FAD and phenol in the template structure.

Nomenclature—Subscript letters indicate the substrate on which the mutant was screened (G, guaiacol; T, 2-*tert*-butylphenol); numbers indicate the round of error-prone PCR. HbpA* represents HbpA variants in general.

RESULTS

Directed Evolution of HbpA—2-Hydroxybiphenyl 3-monooxygenase (HbpA) was subjected to *in vitro* manganese mutagenesis with error-prone PCR (41) and subsequent *in situ* screening for enzymes with an altered substrate reactivity (HbpA*). The base substitution rate in the error-prone PCR was tuned to an exchange rate of 1–3 per *hbpA* gene, to produce an average of one amino acid substitution per HbpA* (24). The Mn^{2+}

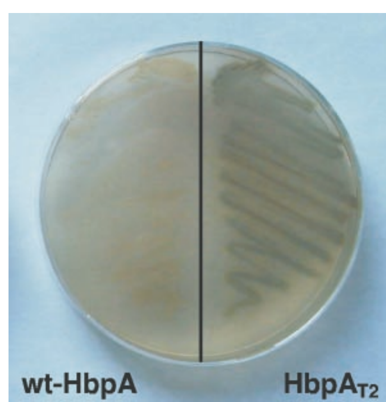

FIG. 2. ***E. coli* JM101 synthesizing HbpA and HbpA$_{T2}$ on LB medium containing 0.5 mM 2-*tert*-butylphenol.** 2-*tert*-Butylphenol was directly added to the medium, and cells were allowed to grow overnight at 30 °C. Time and intensity of the reddish color formation, which results from 3-*tert*-butylcatechol polymerization, was used to select enzymes with different substrate reactivities.

concentration was adapted to template composition, template length, dNTP concentration, and polymerase type. We tested different Mn^{2+} concentrations in a range of 0.1–1 mM. At a concentration of 0.5 mM Mn^{2+}, 60–70% of the amplified *hbpA* genes encoded for active HbpA. Sequencing of randomly picked active or inactive clones showed that on average there were 1.2 amino acid substitutions per HbpA*. With respect to the base substitutions, transitions exceeded transversions by a factor of 2.

The mutant library was plated on substrate-containing medium, where active HbpA produces aromatic polymers. The improvement of enzyme activity is generally associated with a decrease of the K_m towards the substrate (24). We used aromatic substrate concentrations that were lower than the K_m of the parent enzyme. The color of the polymer formed depended on the screening substrate used; the polymer formed from 3-methoxycatechol was brownish, whereas the 3-*tert*-butylcatechol polymer was reddish. Fig. 2 shows *E. coli* JM101 growing on solidified LB medium containing 0.5 mM 2-*tert*-butylphenol and expressing either wild-type *hbpA* or *hbpA*$_{T2}$.

After the first round of mutagenesis, active clones were screened for increased activity on guaiacol and 2-*tert*-butylphenol. From each experiment (500 clones), we took 6–8 clones, which showed increased color formation, and determined NADH oxidation in crude cell extracts as a function of the test substrate and the physiological substrate 2-hydroxybiphenyl. In cases where the activity towards the test substrate or the ratio between the activities for the test substrate and 2-hydroxybiphenyl was higher than for the parent enzyme, product formation was analyzed by reverse phase HPLC.[1] The levels of HbpA and HbpA* were checked with SDS-PAGE to correct for different expression levels.

Eight clones were initially selected following *in situ* screening on guaiacol. In five cell extracts, the recombinant protein level was increased, but the specific HbpA* activity remained constant. Two cell extracts contained HbpA* with a lower *in vitro* activity than the wild type enzyme. Sequencing revealed that both enzymes contained one amino acid substitution, which probably decreased the enzyme stability *in vitro* but not *in vivo*. One mutant monooxygenase was found to have an increased specific activity towards guaiacol and was named HbpA$_{G1}$.

Six clones were initially selected following *in situ* screening on 2-*tert*-butylphenol. Only one clone harbored an *hbpA** gene

[1] The abbreviation used is: HPLC, high pressure liquid chromatography.

TABLE I
Amino acid substitutions in HbpA mutants

Enzyme	Amino acid position		
	244	368	417
HbpA	Ile	Val	Leu
HbpA$_{G1}$	Ile → Val	Val	Leu
HbpA$_{T1}$	Ile	Val → Ala	Leu
HbpA$_{T2}$	Ile	Val → Ala	Leu → Phe

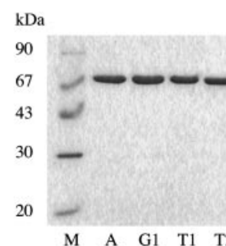

FIG. 3. **SDS-PAGE of purified HbpA and mutants.** Coomassie Blue-stained SDS-polyacrylamide gel containing 4 μg of protein per lane. Proteins were purified from a recombinant *E. coli* JM101, which expressed either the *hbpA* or *hbpA** gene. *Lane M*, marker; *lane A*, HbpA; *lane G1*, HbpA$_{G1}$; *lane T1*, HbpA$_{T1}$; *lane T2*, HbpA$_{T2}$.

with a base substitution that led to an amino acid exchange and a higher activity towards this substrate. This gene (*hbpA*$_{T1}$) was used for another round of error-prone PCR and *in situ* screening. From two selected clones, we obtained one mutant monooxygenase with a higher activity towards 2-*tert*-butylphenol. This variant was named HbpA$_{T2}$.

Purification and Characterization of Mutant Enzymes—The *hbpA*$_{T1}$ and the *hbpA*$_{G1}$ genes each contained one and the *hbpA*$_{T2}$ gene contained two base substitutions that led to an amino acid change (Table I). In addition, *hbpA*$_{T1}$ and *hbpA*$_{T2}$ each carried one silent mutation, and *hbpA*$_{G1}$ carried 2 base substitutions that did not result in an amino acid change.

Wild-type and mutant HbpA were purified from recombinant *E. coli* JM101 harboring a pUC18 derivative carrying the corresponding *hbpA* gene. Using this system, HbpA and HbpA* could be overexpressed to about 20% of total cell protein. The enzymes were purified as tetramers to homogeneity with yields around 30%. Purity was confirmed by SDS-PAGE (Fig. 3), which showed only HbpA or HbpA* monomers.

The mutant enzymes followed Michaelis-Menten kinetics with all substrates tested as does wild-type HbpA. For K'_m determinations, HbpA and HbpA* activities were measured by NADH oxidation at different substrate concentrations. All mutants showed an increased K'_m towards the natural substrate 2-hydroxybiphenyl, whereas K'_m remained unchanged for 2-*sec*-butylphenol. The K'_m towards guaiacol was significantly decreased for all mutants (Table II).

Because HbpA and HbpA* showed uncoupling of NADH oxidation from substrate hydroxylation for all substrates tested, apparent turnover rates were determined by measuring product formation and/or substrate consumption with reverse phase HPLC (Table III). Using this method, HbpA$_{G1}$ showed a 30% increased specific activity towards 2-hydroxybiphenyl, 2-*sec*-butylphenol, and guaiacol. Compared with HbpA, HbpA$_{T2}$ showed half the activity towards 2-hydroxybiphenyl and a 12% lower activity towards 2-*sec*-butylphenol. At the same time, it revealed a 5-fold increase in activity towards 2-*tert*-butylphenol, the substrate on which the enzyme was screened, and twice the activity of HbpA towards guaiacol and salicylaldehyde.

Uncoupling of NADH Oxidation and Substrate Hydroxylation—The NADH oxidase activity of the mutant enzymes was determined spectrophotometrically in the absence of the aromatic substrate. At saturating concentrations of the coenzyme

(6 mM), HbpA showed an NADH oxidase activity of 0.11 units mg^{-1} protein. The activity of the mutants was significantly higher and found to be 0.19 units mg^{-1} protein for $HbpA_{G1}$ and 0.29 units mg^{-1} protein for $HbpA_{T2}$, respectively.

In the presence of the aromatic substrate, the rate of NADH oxidation by HbpA or HbpA* generally exceeds the rate of substrate consumption, due to uncoupling of NADH oxidation from substrate hydroxylation (Table IV), thus lowering the hydroxylation efficiencies of these enzymes. Interestingly, whereas the hydroxylation efficiencies of HbpA and $HbpA_{G1}$ were similar for each of the substrates tested, it was considerably higher for $HbpA_{T1}$ and $HbpA_{T2}$. The uncoupling of substrate hydroxylation from NADH oxidation can have different origins (1, 3). One possibility is that substrate is bound to the enzyme but not hydroxylated due to inefficient oxygen transfer from the flavin (C4a)-hydroperoxide. Alternatively, product remains bound to the enzyme and can not be hydroxylated but can induce the elimination of hydrogen peroxide (Fig. 4). To distinguish between substrate- and product-related uncoupling for HbpA and $HbpA_{G1}$, oxygen consumption was monitored in activity assays in the absence and in the presence of catalase. Whereas for 2-*sec*-butylphenol and guaiacol, most uncoupling could be ascribed to the substrates, for 2-hydroxybiphenyl more than half of the uncoupling could be attributed to the product 2,3-dihydroxybiphenyl. This was confirmed by incubating the enzymes with the reaction product 2,3-dihydroxybiphenyl, which resulted in an NADH oxidase activity of 1.9 units mg^{-1} protein for HbpA and 2.0 units mg^{-1} protein for $HbpA_{G1}$. With both enzyme variants, 2,3-dihydroxybiphenyl acted as a true nonsubstrate effector, since no product formation could be detected. In contrast, 3-*sec*-butylcatechol and 3-methoxycatechol hardly stimulated NADH oxidation in wild-type HbpA or any of the mutants.

Structure Homology Modeling—To assess the effects of the amino acid replacements in the mutant enzymes, a sequence alignment between HbpA (586 residues) and phenol 2-monooxygenase from *T. cutaneum* (PHHY; 664 residues), the most closely related enzyme with known three-dimensional structure (42), was performed. Fig. 5 shows the alignment with the three conserved sequence motifs with a putative dual function in FAD/NAD(P)H binding (2). Most sequence homology between HbpA and PHHY was found in the N-terminal part of the proteins, which consists of the FAD-binding and substrate-binding domains and constitutes the enzyme active site. A sequence identity of 24.4% was calculated when only these parts of HbpA and PHHY were taken into account. There was less homology in the C-terminal part of the proteins, reducing the overall sequence identity to 20.1%. The only known function of the C-terminal domain of PHHY is its participation in subunit association (42).

Structure homology modeling with PHHY confirmed that HbpA consists of three domains. The FAD-binding and substrate-binding domains of both enzymes are structurally conserved, but the structure of the C-terminal domain of HbpA is more uncertain. Like *p*-hydroxybenzoate hydroxylase, which

TABLE II

Apparent K_m values of HbpA and mutants towards different 2-substituted phenols

Apparent K_m values were determined by spectrophotometrically monitoring NADH consumption. The assays were performed at 30 °C in 20 mM phosphate buffer (pH 7.5) with 0.3 mM NADH and the following substrate concentrations: 2-hydroxybiphenyl and 2-*sec*-butylphenol: 2, 3, 4, 5, 10, 15, 20, and 25 μM; guaiacol: 0.025, 0.05, 0.1, 0.2, 0.3, 0.4, 0.5, and 0.8 mM.

R[a]	K_m[b]			
	HbpA	$HbpA_{G1}$	$HbpA_{T1}$	$HbpA_{T2}$
	μM			
Phenyl	2.6 ± 0.1	5.7 ± 0.1	13 ± 1.6	16 ± 1.4
sec-Butyl	8.7 ± 0.9	9.5 ± 1.0	ND[c]	10.0 ± 0.2
Methoxy[d]	588 ± 13	222 ± 13	337 ± 58	143 ± 19

[a] Substituent *ortho* to the phenolic hydroxy group.
[b] Best fit parameters obtained from nonlinear least square fits to the Michaelis-Menten model.
[c] Not determined.
[d] Resulting phenol: guaiacol.

TABLE IV

Uncoupling of product formation from NADH oxidation

The assays were performed at 30 °C in 20 mM phosphate buffer (pH 7.5) containing 0.3 mM NADH. Substrate concentrations used were as follows: 2-hydroxybiphenyl, 2-*sec*-butylphenol, 0.2 mM; guaiacol, 1 mM.

	Specific monooxygenase activity		Uncoupling
	NADH oxidation[a]	Product formation[b]	
	$\mu mol\ min^{-1}\ mg\ protein^{-1}$		%
2-Hydroxybiphenyl			
HbpA	3.65	2.89	21
$HbpA_{G1}$	5.41	3.86	29
$HbpA_{T1}$	3.80	3.70	3
$HbpA_{T2}$	1.61	1.55	4
2-*sec*-Butylphenol			
HbpA	4.15	3.39	18
$HbpA_{G1}$	5.77	4.51	22
$HbpA_{T2}$	3.18	2.99	6
Guaiacol			
HbpA	0.98	0.22	78
$HbpA_{G1}$	1.84	0.30	84
$HbpA_{T1}$	0.66	0.31	53
$HbpA_{T2}$	0.88	0.43	51

[a] All values were corrected for the endogenous NADH oxidation and have an S.E. of ≤10%.
[b] Determined by measuring substrate consumption and product formation with reverse phase HPLC.

TABLE III

k_{cat} and k_{cat}/K_m values of HbpA and HbpA mutants towards different 2-substituted phenols

The k_{cat} values were determined for the tetrameric enzyme. The assays were performed at 30 °C in 20 mM phosphate buffer (pH 7.5) containing 0.3 mM NADH. Substrate concentrations used for the determination of k_{cat} were 0.1 mM for 2-hydroxybiphenyl, 2-*sec*-butylphenol and 1 mM for salicylaldehyde, guaiacol, 2-*tert*-butylphenol.

R[a]	HbpA		$HbpA_{G1}$		$HbpA_{T2}$	
	k_{cat}	k_{cat}/K_m	k_{cat}	k_{cat}/K_m	k_{cat}	k_{cat}/K_m
	s^{-1}	$s^{-1}\ \mu M^{-1}$	s^{-1}	$s^{-1}\ \mu M^{-1}$	s^{-1}	$s^{-1}\ \mu M^{-1}$
Phenyl[b]	11.9	4.6	16.2	2.8	6.4	0.4
sec-Butyl[b]	14.5	1.7	19.1	2.0	12.8	1.3
Methoxy[b]	0.95	1.6×10^{-3}	1.28	5.8×10^{-3}	1.83	1.3×10^{-2}
Formyl[b]	0.5	ND[c]	0.4	ND	0.9	ND
tert-Butyl[d]	<0.1	ND	<0.1	ND	0.5	ND

[a] Substituent *ortho* to the phenolic hydroxy group.
[b] Activities determined by measuring product formation and substrate consumption with reverse phase HPLC.
[c] ND, not determined.
[d] Activities determined by measuring substrate consumption with reverse phase HPLC.

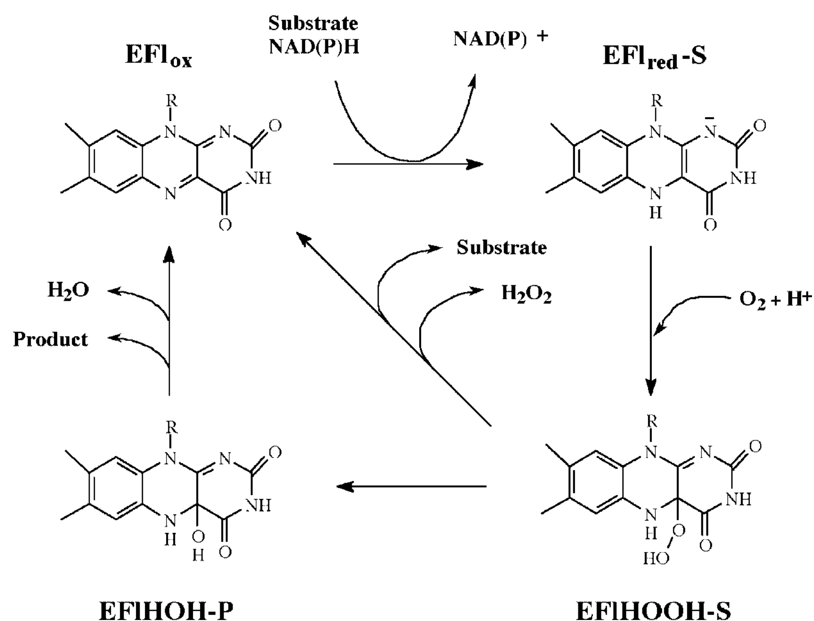

FIG. 4. **Reaction cycle of flavoprotein aromatic hydroxylases.** Reaction cycle of flavoprotein aromatic hydroxylases adapted from van Berkel *et al.* (45). *EFl$_{ox}$*, enzyme containing oxidized flavin; *EFl$_{red}$-S*, reduced flavin enzyme-substrate complex; *EFlHOOH-S*, flavin C(4a)-hydroperoxide enzyme-substrate complex; *EFlHOH-P*, flavin C(4a)hydroxide enzyme-product complex.

contains no extra domain (43, 44), bacterial HbpA contains considerably fewer surface loops than eukaryotic PHHY. Fig. 6 shows a model of the HbpA subunit, as obtained by using the "closed" subunit of PHHY (Protein Data Bank entry 1foh) as the template file. For PHHY it was reported that the two subunits in the homodimer do not have an identical conformation and that the largest difference involves a loop (residues 170–210, PHHY numbering) which can act as a lid that opens and closes the active site (42). In HbpA, this active site loop (residues 142–164) is much shorter, but the model suggests that it is still able to cover the active site.

Further examination of the three-dimensional model of HbpA revealed that the amino acids Ile^{244}, Val^{368}, and Leu^{417} that were changed in the variants are spread throughout the structure. Ile^{244} is located in the substrate binding pocket and is rather close to the flavin ring. Interestingly, Ile^{244} corresponds with Tyr^{289} of PHHY. This tyrosine is believed to play an important role in catalysis by positioning the aromatic substrate for attack at the *ortho* position (42). Val^{368} and Leu^{417} are both located near the protein surface of the HbpA subunit, and Leu^{417} is positioned far away from the substrate binding site. Val^{368} corresponds with Ile^{414} in PHHY and is part of a conserved helix, whereas Leu^{417} corresponds with Val^{480} in PHHY and is located in the beginning of the C-terminal domain. Although Ile^{414} and Val^{480} are far away from the dimer interface of PHHY, the possibility cannot be excluded that in HbpA, Val^{368} and Leu^{417} play a role in tetramer formation or tetramer stabilization.

DISCUSSION

Most members of the family of flavoprotein hydroxylases are involved in the degradation of aromatic compounds by soil microorganisms (45). However, the application of these redox enzymes is not restricted to the metabolism of pollutants in our environment. Due to their high regioselectivity, flavoprotein hydroxylases also have considerable potential in the synthesis of new fine chemicals.

Directed Evolution of HbpA—Assuming an electrophilic aromatic substitution reaction mechanism (3), we chose 2-*tert*-butylphenol and guaiacol (2-methoxyphenol) as model substrates for directed evolution of HbpA. These substrates differ significantly from the natural substrate: (i) the bulky side chain of 2-*tert*-butylphenol requires more room in the enzyme active site, and (ii) the methoxy group of guaiacol is more polar and withdraws more charge from the aromatic system (+M, −I compared with +M, +I) (46). Furthermore, so far no activity for the *ortho*-hydroxylation of guaiacol and 2-*tert*-butylphenol has been described. Microbial degradation of guaiacol proceeds only via demethylation (47–49), whereas chlorinated guaiacols can also be degraded via *para*-hydroxylation (50, 51). Thus, HbpA variants with an increased catalytic activity towards guaiacol and 2-*tert*-butylphenol will allow the biotechnological production of the corresponding catechols and may yield information about the structure-function relationship of HbpA.

The key factor for a successful directed evolution experiment is an effective screening or selection procedure (24, 52). With HbpA, the instability of the formed 3-substituted catechols offered a good basis for the development of a suitable *in situ* screening procedure. The time-dependent color formation could be efficiently used for qualitative estimation of enzyme activity directly after construction of the mutant library. The high reliability of our *in situ* screening procedure was illustrated by the fact that only 2–8 clones per round of mutagenesis had to be selected to obtain a successfully modified enzyme. Furthermore, the *in situ* screening was not restricted to the directed evolution of HbpA towards 2-*tert*-butylphenol. A second mutant library was screened on guaiacol, and enzymes with an increased activity towards this substrate could also be selected. This demonstrates that *in situ* screening provides an easy and rapid method for the detection of specific enzyme features if the corresponding assay can be applied on solid media.

Enzyme Kinetics—The mutant proteins, which were selected for higher activity towards 2-*tert*-butylphenol and guaiacol, were characterized with respect to their catalytic properties and substrate specificity. Mutations V368A/L417F changed the substrate reactivity of HbpA for the hydroxylation of different 2-substituted phenols, whereas mutation I244V reduced the substrate spectrum but increased the turnover rate. Interestingly, HbpA$_{G1}$ (I244V) showed an increased activity with guaiacol but not towards salicylaldehyde, whereas HbpA$_{T2}$ (V368A/L417F) showed a doubled activity towards both phenols. This suggests that the substrate side chain causes mostly steric rather than inductive effects. This conclusion is supported by the results obtained with 2-*sec*-butylphenol. This substrate has a flexible side chain, which can move freely in several directions. This results in an approximately equal activity and K'_m values for the wild-type enzyme and all mutants.

```
HbpA    1 MSNSAETDVLIVGAGPAGAMSATLLASLG-----IRSLMINRWRSTSPGPRSHIINQRTMEILRDIGLEESAKSLAVPKEYMGEH   80
              +  DVLIVGAGPAG M+A +L+          ++  +I++  +       ++  +  RT+E L+++GL +    S A       +  +
PHHY    1 TKYSESYCDVLIVGAGPAGLMAARVLSEYVRQKPDLKVRIIDKRSTKVYNGQADGLQCRTLESLKNLGLADKILSEANDMSTIALY   86
            < βA > <βB>   <    α1         >     <βA>                   <   α2    > <  α3 >     < βC
                VhhhGsGhhGhhhs
               FAD fingerprint (1)

HbpA   81 VYATSLAGEEFGRIPAWAS----------HPQAHAEHELASPSRYCDLPQLYFEPMVVSEAALRG------------ADVRFLTEY  144
                      RIP                H      + L S +    D      E P++       +                 +R+++E+
PHHY   87 NPDENGHIRRTDRIPDTLPGISRYHQVVLHQGRIERRILDSIAEISDTRIKVERPLIPEKMEIDSSKAEDPEAYPVTMTLRYMSED  172
           >     <  βC  >                <     α4        >          <  βA     ><α5>         <  βA  >  <α

HbpA  145 LG------------------HVEDQDGVTARLLD-HVSGAEYEVRAKYIIGADGAHSLVAQNAGLPFEGQMGIGDSGSINIEFSAD  211
                                ++++     RL +    +G     V   KY+IG DG HS V +  G     G+        G ++     +++
PHHY  173 ESTPLQFGHKTENGLFRSNLQTQEEEDANYRLPEGKEAGEIETVHCKYVIGCDGGHSWVRRTLGFEMIGEQTDYIWGVLDAVPASN  258
          6>                   <   α7   >                 < βA > <βB>        < α8 > <βD>   <  βC  ><βE>
                                                            chhhssDGxcSxhR
                                                           conserved motif

HbpA  212 LSSLCEHRKGDMYWMFRAGSGINGVGVAALRMIRPWNKWICV-WGYEKSKGTPEITKEEAKKIIHEIIGTDEIPVEVGPISTWTIN  296
             +    +  ++          I          +R                ++++K TPE+   +AKKI H              +    + + I
PHHY  259 FPDIRS--RCAIHSAESGSIMIIPRENNLVRFYVQLQARAEKGGRVDRTKFTPEVVIANAKKIFHPYT---FDVQQLDWFTAYHIG  339
          >       <βC >   <  βC  >     <  βC >                  <      α9     >      <βE>  <  βC   ><

HbpA  297 QQYAVRNTSG-RVFCMGDAVHRHTPMGGLGLNTSVQDAYNLAWKLALVLKGTAAPTLLDSYDAERSPVAKQIVERAFKS-------  374
          Q+    + +   RVF  GDA H H+P   G G NTS+ D YNL WKL LVL G A    +L +Y+ ER+P A  +++
PHHY  340 QRVTEKFSKDERVFIAGDACHTHSPKAGQGMNTSMMDTYNLGWKLGLVLTGRAKRDILKTYEEERQPFAQALIDFDHQFSRLFSGR  425
          βD>         <βB><α10>        <        α11         >      <               α12             >
                   GxxhhLhGDAAHxxxPxxGxGxNxsxxDsxxL
                        FAD fingerprint (2)

HbpA  375 ------------LSTFPPVFEALSLPPAPTESEMAEALVRLKDASEEGAKRRAALRKAMDAT-IIGLGGGHGVELNQRYVSR----  443
                      +  F   F        + T  +  E LV  K +S +    +    +     +  +    G  +    R V+
PHHY  426 PAKDVADEMGVSMDVFKEAFVKGNEFASGTAINYDENLVTDKKSSKQELAKNCVVGTRFKSQPVVRHSEGLWMHFGDRLVTDGRFR  511
                      <      α13       >                  <α14>         <βF>   <βF><α15>     <

HbpA  444 -AVFP----DGTPDPGFVRDQEFFYQASTRPGAHLPHVWLTENQRRISTLDLCGKGRFTLLTGLS---GAAWKH-----EAEQVSQ  516
            VF     D T      +    +      +     + P +   + +   + T+  C +    +     +         W +      + +
PHHY  512 IIVFAGKATDATQMSRIKKFAAYLDSENSVISRYTPKGADRNSRIDVITIHSCHRDDIEMHDFPAPALHPKWQYDFIYADCDSWHH  597
           βG  >    <     α16      >   <α17>           <  βG  >      <α18>            <βG>

HbpA  517 SLGIELKVCVIGPGQEFVDTYGEYAKISEIGESGALLVRPDMFIAFRAKDASREGLEQLNVAVKSILGRA             586
                  +       V           S + +            F            + G +       KS
PHHY  598 PHPKSYQAWGVDETKGAVVVVRPDGYTSLVTDLEGTAEIDRYFSGILVEPKEKSGAQTEADWTKSTA                664
              <α19>    <βG>     <βG  >  <   α20   >
```

FIG. 5. **Alignment of 2-hydroxybiphenyl 3-monooxygenase (HbpA) from *P. azelaica* HBP1 and phenol 2-monooxygenase (PHHY) from *T. cutaneum* containing the conserved sequence motifs of flavoprotein aromatic hydroxylases.** *Letters* and *symbols between* the two sequences represent identical (*letters*) and similar (+) residues. The consensus profiles according to Eppink *et al.* (2) shown *below* the alignment include strictly conserved residues in *boldface letters*. *Uppercase letters* are amino acid residues. *Lowercase letters* are as follows: hydrophobic residues (*h*); small residues (*s*); charged residues (*c*); all residues (*x*). -, gap. The *shaded boxes* mark the location of the amino acid substitutions in the HbpA mutants.

Most enzymes in nature do not function under V_{max} conditions but catalyze reactions at [S]/K_m ratios between 0.01 and 0.1 (53). The catalytic efficiency under these conditions is described by the ratio k_{cat}/K_m. This ratio was determined for HbpA and its variants. K'_m values for all mutant enzymes were decreased compared with wild-type HbpA towards guaiacol but increased for the physiological substrate 2-hydroxybiphenyl. This was even the case for $HbpA_{G1}$, which has an increased activity towards 2-hydroxybiphenyl. This suggests that evolutionary advantage can be more easily achieved with a low K_m rather than a high activity, probably because environmental substrate concentrations are low.

A low K_m for the substrate of interest is also important for the biotechnological production of 3-substituted catechols with HbpA variants. The substituted phenols as well as the formed catechols are highly bactericidal and inactivate the whole-cell biocatalyst. However, this problem can be solved by processes with integrated *in situ* product recovery or two-liquid-phase bioconversions (13, 54, 55). These processes are based on the principle that both substrate and product are present at low concentrations in the aqueous phase of the bioreactor. Therefore, a desired enzyme feature for these processes is a high activity at the lowest possible substrate concentration (*i.e.* a low K_m).

Location of Mutations in the HbpA Model—Lacking information on the three-dimensional structure of HbpA, an interpretation of the structural effects of the obtained amino acid substitutions is speculative. However, structure homology modeling with PHHY allows some statements on the effects of the modifications in the HbpA variants. Interestingly, Ile^{244} of HbpA corresponds with Tyr^{289} of PHHY (42) and Tyr^{222} of *p*-hydroxybenzoate hydroxylase (56, 57). In *p*-hydroxybenzoate hy-

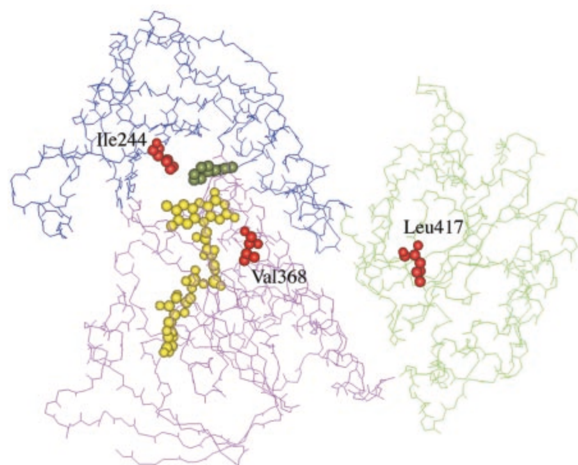

FIG. 6. **Three-dimensional model of a subunit of HbpA.** The closed subunit conformation of phenol 2-monooxygenase from *T. cutaneum* served as template. The substrate binding domain is drawn in *blue*, the FAD binding domain in *magenta*, and the C-terminal domain in *green*. Highlighted are the substrate 2-hydroxybiphenyl (*olive*), the FAD prosthetic group (*yellow*), and the amino acids (*red*) that were changed during the directed evolution experiment.

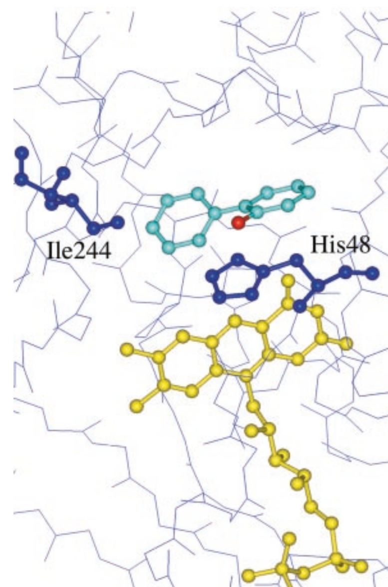

FIG. 7. **Model of the substrate binding pocket of HbpA.** *Highlighted* are the FAD prosthetic group (*yellow*), the substrate 2-hydroxybiphenyl (*light blue*), and amino acids His[48] and Ile[244] (*dark blue*).

droxylase, Tyr[222] interacts with the carboxylic moiety of the substrate and is critically involved in closing the active site, allowing efficient substrate hydroxylation (for a review, see Ref. 58). In PHHY, Tyr[289] has been shown to play an important role in orienting the substrate for attack at the *ortho* position by forming a hydrogen bond with the hydroxyl moiety of the phenol. Moreover, Tyr[289] favors the flavin out rather than the flavin in position through formation of a hydrogen bond between the N-3 of the isoalloxazine ring and the phenolic hydroxyl group (35). In HbpA, Ile[244] clearly cannot fulfill a similar function. However, its position close to the side chain of the phenolic substrate suggests a direct influence on the shape of the substrate binding pocket (Fig. 7). From the above considerations and the catalytic properties of the I244V variant, we suggest that substitution of Ile[244] by Val in HbpA has a small but significant effect on substrate binding due to the reduced size of the side chain.

Although the mode of binding of 2-hydroxybiphenyl in HbpA is unknown, it is reasonable to assume that it resembles the mode of binding of phenol in PHHY. This assumption is based on the conserved mode of binding of the FAD, the similar hydrophobic nature of the substrate binding pockets, and the fact that the active site base Asp[54] of PHHY is replaced by His[48] in HbpA (Fig. 7). This histidine is likely to play an essential role in activating the 2-hydroxybiphenyl molecule prior to the regiospecific electrophilic attack by the flavin (C4a)-hydroperoxide at the 3-position of the phenolic ring.

Uncoupling of NADH Oxidation from Substrate Hydroxylation—A common feature among flavoprotein aromatic hydroxylases is the uncoupling of substrate hydroxylation from NADH oxidation with the concomitant formation of hydrogen peroxide (59). This is also observed during hydroxylation of 2-hydroxybiphenyl with HbpA (1). Mutation I244V in $HbpA_{G1}$ had no significant influence on the degree of uncoupling compared with the wild-type enzyme, but the mutations V368A/L417V in $HbpA_{T2}$ decreased the uncoupling with all substrates. This is a remarkable result, because both these substitutions are located far away from the substrate binding site. From the properties of the single mutant $HbpA_{T1}$, it can be concluded that the improvement of the efficiency of hydroxylation is related to the V368A substitution. This amino acid is located in the FAD binding domain, which suggests an effect on the mobility of the flavin ring. Whether this results in a stabilization and/or improved positioning of the flavin (C4a)-hydroperoxide towards the substrate remains to be investigated. The mutation L417V is located in the third domain, which in the case of PHHY is thought to be involved in subunit interactions (42). The increased hydroxylation efficiency due to substitution V368A remained, but the activity towards 2-hydroxybiphenyl decreased significantly. In combination with the increased K'_m and higher k'_{cat} for guaiacol, we conclude that the altered catalytic properties of L417V are due mainly to a changed substrate binding. Detailed structural information will be necessary to understand how the substitutions effect this change. Clearly, given their location in HbpA, substitutions V368A and L417V would not have been obvious targets for rational protein design.

For wild-type HbpA and $HbpA_{G1}$ with 2-hydroxybiphenyl as the substrate, uncoupling could be ascribed for more than 50% to the reaction product 2,3-dihydroxybiphenyl. This suggests that 2,3-dihydroxybiphenyl competes with 2-hydroxybiphenyl for binding to the reduced enzyme and induces the nonproductive heterolytic cleavage of the flavin (C4a)-hydroperoxide. This interpretation is supported by results from rapid reaction kinetics studies (3). In contrast, 3-*sec*-butylcatechol and 3-methoxycatechol have only minor effects on the total uncoupling of wild-type HbpA and $HbpA_{G1}$, indicating that these aromatic products do not interact strongly with the reduced enzyme.

Flavoprotein aromatic hydroxylases such as *p*-hydroxybenzoate hydroxylase have a mechanism to decrease the rate of flavin reduction by several orders of magnitude in the absence of an aromatic substrate, thereby preventing the wasteful consumption of NAD(P)H (60–62). Other flavin enzymes such as 4-hydroxyphenylacetate 3-hydroxylase, PHHY, and HbpA are less efficient in this respect and show some residual NAD(P)H oxidation (1, 63, 64). For HbpA, it has been shown by stopped-flow absorption spectroscopy that flavin reduction is the rate-limiting step in this NADH oxidation (3). An increased NADH oxidase activity of the substrate-free enzyme, as determined for all HbpA mutants, is therefore most likely related to an increase in the rate of flavin reduction.

In conclusion, we have shown that the catalytic properties and substrate reactivity of HbpA can be improved by random mutagenesis. This is the first successful modification of a flavin-dependent monooxygenase by molecular evolution. We expect the mutants to be useful in new biocatalytic processes and the

insights obtained to be helpful in further investigations of structure-function relationships of flavin monooxygenases.

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