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

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The substrate reactivity of the flavoenzyme 2-hydroxybiphenyl 3-monoxygenase (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-monoxygenase 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-monoxygenase (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, 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 derivative harboring the hbpA gene as a SalI/BstI fragment cloned into the SalI/BstI 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-butylicatetechol 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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Directed Evolution of 2-Hydroxybiphenyl 3-Monoxygenase

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

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

**Directed Evolution of HbpA**—2-Hydroxybiphenyl 3-monoxygenase (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+} (cell weight) of frozen E. coli JM101, harboring a pUC19 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 Amicon) at 70 bars, followed by ultracentrifugation at 4°C (Beckman L8-60, 30,000 × g). 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 DMAG-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. NADH oxidase activity was 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) and Coomassie blue staining.

**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, 0.5 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 dialyzed for 1 h with MeOH, 0.5% phosphoric acid and 200 mM sodium hydroxide, then with another 200 mM sodium hydroxide, 0.5% phosphoric acid and distilled water 3 times. The resulting aqueous layers were analyzed with a Dionex HPLC system (model 1000, Dionex) using a PA100 column (4.6 × 250 mm, 5 μm particles, 80 Å pore size, 300 Å wall thickness; Macherey-Nagel, Düren, Germany). The temperature was controlled at 40°C.

**RESULTS**

Directed Evolution of HbpA—2-Hydroxybiphenyl 3-monoxygenase (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+}
Directed Evolution of 2-Hydroxybiphenyl 3-Monoxygenase

**TABLE I**

| Enzyme       | Amino acid position |
|--------------|---------------------|
| HbpA         | 244                 |
| HbpA<sub>T1</sub> | 368                 |
| HbpA<sub>T2</sub> | 417                 |

**FIG. 2.** *E. coli* JM101 synthesizing HbpA and Hbp<sub>T2</sub> 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<sup>2+</sup> concentrations in a range of 0.1–1 mM. At a concentration of 0.5 mM Mn<sup>2+</sup>, 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<sup>*</sup>. 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<sub>m</sub> towards the substrate (24). We used aromatic substrate concentrations that were lower than the K<sub>m</sub> of the parent enzyme. The color of the polymer formed depended on the screening substrate used; the polymer formed from the parent enzyme. The color of the polymer formed depended on the screening substrate used; the polymer formed from the parent enzyme.

**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<sup>*</sup> gene. Lane M, marker; lane A, HbpA; lane G1, HbpA<sub>T1</sub>; lane T1, HbpA<sub>T1</sub>; lane T2, HbpA<sub>T2</sub>.

with a base substitution that led to an amino acid exchange and a higher activity towards this substrate. This gene (hbpA<sub>T1</sub>) 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<sub>T2</sub>.

**Purification and Characterization of Mutant Enzymes**—The hbpA<sub>T1</sub> and the hbpA<sub>G1</sub> genes each contained one and the hbpA<sub>T2</sub> gene contained two base substitutions that led to an amino acid change (Table I). In addition, hbpA<sub>T1</sub> and hbpA<sub>T2</sub> each carried one silent mutation, and hbpA<sub>G1</sub> 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<sup>*</sup> 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<sup>*</sup> monomers.

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

Because HbpA and HbpA<sup>*</sup> 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<sub>G1</sub> showed a 30% increased specific activity towards 2-hydroxybenzyl, 2-sec-butylphenol, and guaiacol. Compared with HbpA, HbpA<sub>T2</sub> showed half the activity towards 2-hydroxybenzyl 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 HbpAG1 and 0.29 units mg\(^{-1}\) protein for HbpAT2, respectively.

In the presence of the aromatic substrate, the rate of NADH oxidation by HbpA or HbpA* generally exceeds the rate of substrate hydroxylation from NADH oxidation (Table IV), thus lowering the substrate consumption, due to uncoupling of NADH oxidation by HbpA or HbpA*.

Whereas for 2-hydroxybiphenyl, the oxidation of NADH by HbpA and HbpA* was similar for each of the substrates tested, it was considerably higher for HbpAT1 and HbpAT2. 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 recombination can induce the elimination of hydrogen peroxide (Fig. 4).

To distinguish between substrate- and product-related uncoupling for HbpA and HbpA*, 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 HbpAG1. With both enzyme variants, 2,3-dihydroxybiphenyl acted as a true nonsubstrate effector, since no product formation could be detected.

In contrast, 3-sec-butylecatechol 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-monoxygenase 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 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) containing 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\(^{a}\) | HbpA | HbpAG1 | HbpAT1 | HbpAT2 |
| --- | --- | --- | --- | --- |
| 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\(^{d}\) | 10.0 ± 0.2 |
| Methoxy | 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.  
\(^{c}\) ND: not determined.  
\(^{d}\) Resulting phenol: guaiacol.

| 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\(^{b}\) | HbpA | HbpAG1 | HbpAT1 | HbpAT2 |
| --- | --- | --- | --- | --- |
| Phenyl | 11.9 | 4.6 | 16.2 | 2.8 |
| sec-Butyl | 14.5 | 4.7 | 19.1 | 2.0 |
| Methoxy | 0.95 | 1.6 × 10\(^{-3}\) | 1.28 | 5.8 × 10\(^{-3}\) |
| Formyl | 0.5 | ND | 0.4 | ND |
| tert-Butyl | <0.1 | ND | <0.1 | 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.
Directed Evolution of 2-Hydroxybiphenyl 3-Monoxygenase

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_I244V (244V) showed an increased activity with guaiacol but not towards salicylaldehyde, whereas HbpA_L417F (244V/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.

**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.

Fig. 4. Reaction cycle of flavoprotein aromatic hydroxylases. Reaction cycle of flavoprotein aromatic hydroxylases adapted from van Berkel et al. (45). $EF_{ox}$, enzyme containing oxidized flavin; $EF_{red-S}$, reduced flavin enzyme-substrate complex; $EFIHOH-S$, flavin C(4a)-hydroperoxide enzyme-substrate complex; $EFIHOH-P$, flavin C(4a)hydroxide enzyme-product complex.
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}/H_{11032} 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 HbpAG1, 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 shows that Ile244 of HbpA corresponds to Tyr289 of PHHY (42).
His48 in HbpA (Fig. 7). This histidine is likely to play an ties of the I244V variant, we suggest that substitution of Ile244 by a direct influence on the shape of the substrate binding pocket position close to the side chain of the phenolic substrate suggests HbpA, Ile244 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 Ile244 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 Asp54 of PHHY is replaced by His68 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 HbpAcat had no significant influence on the degree of uncoupling compared with the wild-type enzyme, but the mutations V368A/L417V in HbpAcat 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 HbpAcat, 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, 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 HbpAcat, 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 HbpAcat, 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 monoxygenase 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 monoxygenases.

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