Hydroxylation of Indole by Laboratory Evolved

2-Hydroxybiphenyl 3-Monooxygenase

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Running title:

Indole Hydroxylation by Laboratory Evolved HbpA
SUMMARY

Directed enzyme evolution of 2-hydroxybiphenyl 3-monoxygenase (HbpA, EC 1.14.13.44) from Pseudomonas azelaica HBPI resulted in an enzyme variant (HbpA\textsubscript{ind}) which hydroxylates indole and indole derivatives, such as hydroxyindoles or 5-bromoindole. The wild-type protein does not catalyze these reactions. HbpA\textsubscript{ind} contains amino acid substitutions Asp\textsubscript{222}Val and Val\textsubscript{368}Ala. The activity towards indole hydroxylation was increased 18-fold in this variant. Concomitantly the $K_d$ value towards indole decreased from 1.5 mM to 78 µM. Investigation of the major reaction products of HbpA\textsubscript{ind} with indole revealed hydroxylation at the carbons of the pyrrole ring of the substrate. Subsequent enzyme independent condensation and oxidation of the reaction products lead to the formation of indigo and indirubin.

The activity of the HbpA\textsubscript{ind} mutant monooxygenase towards the natural substrate 2-hydroxybiphenyl was 6 times lower than that of the wild-type enzyme. In HbpA\textsubscript{ind}, there was significantly increased uncoupling of NADH oxidation from 2-hydroxybiphenyl hydroxylation, which could be attributed to the substitution Asp\textsubscript{222}Val. The position of Asp\textsuperscript{222} in HbpA, the chemical properties of this residue, and the effects of its substitution indicate that Asp\textsuperscript{222} is involved in substrate activation in HbpA.
INTRODUCTION

Indole is produced from the aromatic amino acid tryptophane in tryptophanase synthesizing bacteria, such as *Escherichia coli* (1). Enzymes that oxygenate the indole pyrrole ring are easily detectable, because the reaction products are unstable and form pigments. This observation was first made when the naphthalene oxidation genes were expressed in *E. coli*, which resulted in the biosynthesis of indigo (2). Based on these results, and because of its importance as a dye, the biocatalytic production of indigo by naphthalene dioxygenase was for some time a major goal of the biotech industry (3, 4).

Naphthalene dioxygenase was soon found not to be the only enzyme capable of indole oxidation: several other oxygenases that accept indole as a substrate have been identified (5-10). These are either enzymes similar to naphthalene dioxygenase that activate oxygen with iron centers or members of the cytochrome P450 family (6, 11). Although flavin nucleotides may be involved in electron transfer from cofactors in these proteins, none is known to be a flavoprotein oxygenase.

This has changed with our recent finding that flavosystem oxygenases can be modified to accept unnatural substrates. 2-Hydroxybiphenyl 3-monooxygenase (HbpA, EC 1.14.13.44) from *Pseudomonas azelaica* HBP1 is a flavoprotein aromatic hydroxylase that catalyzes the hydroxylation of a variety of 2-substituted phenols to the corresponding catechols (12-14). The mechanism of HbpA has been extensively studied by spectroscopic techniques, which revealed that molecular oxygen is activated via the formation of a flavin (C4a)-hydroperoxide (15), a common intermediate in the reaction cycle of this enzyme family (16). HbpA has a broad substrate spectrum but does not hydroxylate indole (13, 17). Recently we changed the substrate reactivity of HbpA by directed enzyme evolution towards 2-tert-butylphenol, a substrate which is not
converted by the wild type enzyme (18, 19). As a side product of this work, we also obtained a HbpA variant, which we denoted HbpA\textsubscript{ind}, with activity towards the hydroxylation of indole.

In this study we report on the characterization of HbpA\textsubscript{ind} with respect to its catalytic properties. While previous work on indole oxygenating enzymes mainly aimed at the biotechnological production of indigo, we were especially interested in the formation of the byproduct indirubin. Indirubin and its analogues have been identified as potent inhibitors of cyclin-dependent kinases (CDK) (20). The crystal structure of CDK2 in complex with indirubin derivatives showed that indirubin binds to the kinase’s ATP binding site. As a consequence, it inhibits the proliferation of a wide range of cells and belongs to a group of novel anticancer compounds that act on the cell cycle (21).
MATERIALS AND METHODS

Chemicals, Strains, and Plasmids

_**Escherichia coli** JM101 (22) and the pUC18 plasmid (23) were used throughout for cloning and expression of the _hbpA_ gene. Alkaline phosphatase (EC 3.1.3.1) was purchased from Roche Molecular Biochemicals (Basel, Switzerland). Catalase (EC 1.11.1.6) from beef liver and formate dehydrogenase (EC 1.2.1.2) from _Candida boidinii_ were obtained from Fluka AG (Buchs, Switzerland). 4-Hydroxyindole and 5-hydroxyindole were from ICN Biomedicals Inc. (Aurora, USA). Components for complex media were obtained from Difco Laboratories (Detroit, USA). All other chemicals were of purest available quality and obtained from Fluka AG (Buchs, Switzerland).

Directed Evolution of HbpA

Directed evolution of HbpA was done by error prone PCR based on _in vitro_ manganese mutagenesis as described earlier (18). The mutant library was subsequently plated onto LB medium. Cells harboring enzymes with activity towards the hydroxylation of indole formed deep blue colonies.

The single mutant D222V (HbpA<sub>D222V</sub>) was constructed using the QuickChange™ site directed mutagenesis kit from Stratagene (La Jolla, USA).

Protein Synthesis and Purification

Synthesis of wild type HbpA and HbpA<sub>md</sub> was done in recombinant _E. coli_ JM101 using M9 mineral medium and glycerol as carbon source (17). After harvesting the cells the proteins were purified according to the method described recently (18).
Analytical Methods

Determination of Activity towards 2-Hydroxybiphenyl - Activity of wild type HbpA and HbpA_{ind} was determined by measuring substrate consumption and product formation with reverse phase HPLC as described elsewhere (24). The assay contained 0.2 µM HbpA or variant protein, 0.3 mM NADH, 0.2 mM 2-hydroxybiphenyl, and 20 mM air saturated phosphate buffer pH 7.5.

Determination of in vivo Indigo Formation - Activity of recombinant *E. coli* JM101 towards the formation of indigo was determined in 250 ml shaking flasks containing 50 ml LB medium (22). Cultures of *E. coli* JM101 harboring a pUC18 derivative encoding HbpA or HbpA_{ind} were inoculated to an OD_{450} of 0.1. The cultures were incubated at 30°C and vigorously shaken. When the culture color turned olive, samples of 1.1 ml were taken. 100 µl of these were used to determine the cell dry weight (CDW) at 450 nm (25). The remaining 1 ml was centrifuged and the supernatant was carefully removed. Cell associated indigo was extracted with N,N-dimethylformamide (DMF) and quantified at 610 nm (ε_{610} 15900 l mol^{-1} cm^{-1}) (8).

Determination of in vitro Indigo Formation - Activity towards indole was determined using an assay with NADH regeneration by formate dehydrogenase (EC 1.2.1.2, FDH) from *Candida boidinii* (Fig. 1) (26). The assay contained 0.2 µM HbpA or variant, 0.25 U FDH, 160 mM sodium formate, 10 U catalase (EC 1.11.1.6) from beef liver, 0.3 mM NADH, and 2 mM indole in 1 ml 50 mM sodium phosphate buffer pH 7.5. The assay was stopped by the addition of 20 µl 10% (v/v) perchloric acid, and the precipitated proteins were spun down. The pellet and tube associated indigo was extracted with DMF and spectrophotometrically quantified.

Determination of Dissociation Constants - Dissociation constants between the enzymes and substrates were determined by monitoring the absorption changes of the enzyme
bound FAD upon binding of substrate (27). For this 12 µM purified wild type HbpA and HbpA_{ind} were titrated with known concentrations of 2-hydroxybiphenyl or indole and the resulting spectra were recorded using a Varian Cary E1 UV/Vis spectrophotometer. Plotting delta absorbance at a specific wavelength allowed the calculation of the dissociation constants by weighted non-linear regression analysis (Enzfitter, Elsevier-Biosoft, UK).

*High Pressure Liquid Chromatography Mass Spectroscopy (HPLC-MS) Analysis* - Analysis of compounds formed during *in vitro* indigo assays was done with reverse phase HPLC-MS (Hewlett Packard, 1100 MSD). The compounds were separated with a Hypersil ODS column (5 µm, 4.5 x 125 mm) and detected with a diode array detector and a mass spectrometer. Acidified (0.1% formic acid) H_{2}O (solvent A) and 50% methanol/50% acetonitrile (solvent B) were applied as mobile phase according to the following timetable: 0 to 8 min, 85A/15B, flow 1 ml min^{-1}; gradient to 10 min, to 65A/35B, flow 2 ml min^{-1}; to 15 min, 65A/35B, flow 2 ml min^{-1}. Standards for isatin, 4-hydroxyindole, 5-hydroxyindole, 2-indolinone, and indole were commercially available. 3-Indoxyl was prepared by dephosphorylating 3-indoxylphosphate with alkaline phosphatase (EC 1.3.1.3) under anaerobic conditions. HPLC-MS analysis of the formed pigments was done under isocratic conditions at a flow rate of 1 ml min^{-1} with 70% (v/v) methanol as mobile phase for the pigments derived from indole, and 40% (v/v) methanol for the one derived from 4- and 5-hydroxyindole.

*Thin Layer Chromatography (TLC) Analysis* - The formed pigments were analyzed by TLC using silica gel cards and either toluene-acetone (4:1) or chloroform-acetone (97:3) as mobile phase (28).

*Electron Microscopy* - For ultrathin sectioning, the cells were fixed in 2.5% glutaraldehyde for 60 min and subsequently washed with water and embedded in low-
melting-point agarose. After fixation in 1% OsO₄ for 60 min the blocks were dehydrated with ethanol and acetone and embedded in Epon-Araldit (29). Sections cut from the Epon-Araldit preparation were contrasted with uranyl acetate and lead citrate.

Freeze fracturing was carried out following the standard procedures using a Balzers BAF 300 apparatus (Balzers-Union Inc., Balzers, Liechtenstein). The specimen sandwiches were fractured at -150°C and immediately replicated with platinum-carbon. All pictures were taken with a Philips EM301 electron microscope.
RESULTS

Directed Evolution of HbpA

We recently changed the substrate reactivity of 2-hydroxybiphenyl 3-monooxygenase (HbpA) from Pseudomonas azelaica HBP1 by directed evolution (18). This work led to a mutant monooxygenase with increased activity towards the hydroxylation of indole. The HbpA variant was denoted HbpA<sub>mut</sub>. E. coli JM101 cultures synthesizing HbpA<sub>mut</sub> turned deep blue when grown overnight on LB medium. Electron microscopy revealed the extracellular accumulation of material, which we believe, consists of the water insoluble pigment. After centrifugation the pigment was extracted from the pellet with N,N-dimethylformamide (DMF). It was authenticated as indigo by thin layer chromatography (TLC) with toluene-acetone (4:1) as the mobile phase and commercially available indigo as standard. This analysis also revealed the presence of a major byproduct. The R<sub>f</sub> value of this red pigment corresponded to the R<sub>f</sub> value determined earlier for indirubin (28). Analysis by HPLC-MS with 70% (v/v) methanol as mobile phase showed two prominent molecular ion (MH<sup>+</sup>) peaks at m/z 263 with retention times of 3.7 and 4.2 min. The UV/Vis spectra and the fragmentation patterns were compared with literature data (28, 30, 31), which confirmed that these two compounds were indigo (3.7 min) and indirubin (4.2 min).

The formation of indigo by recombinant E. coli JM101 growing on LB medium was quantified. Cultures expressing the hbpA<sub>mut</sub> gene accumulated 150 µM indigo within 8 hours, while cultures of the host synthesizing HbpA remained colorless (Fig. 2). Recombinant protein levels in both cultures were checked by SDS-PAGE and HbpA levels were determined to be in the same range of about 20% of total cell protein.
Stability of Biotechnologically Produced Indigo

When pigments were extracted with DMF from recombinant *E. coli* JM101 cultures, the extract had a deep blue color. The blue color disappeared upon storage at room temperature and the solution turned red (Fig. 3). The red pigment was analyzed by UV/Vis spectroscopy, HPLC-MS, and TLC. It was authenticated as indirubin by comparison of the obtained results with literature data (28, 30, 31). Buffering the pH at a value of 7 or acidification with 0.1% (v/v) 10 M hydrochloric acid stabilized the formed indigo while the addition of 0.1% (v/v) 10 M sodium hydroxide or heating accelerated the disappearance of the blue color.

General Properties of HbpA<sub>ind</sub>

The mutant monoxygenase HbpA<sub>ind</sub> differs from wild-type HbpA by two amino acids: Asp<sup>222</sup> was substituted by valine and Val<sup>368</sup> was substituted by alanine. HbpA<sub>ind</sub> was purified according to the procedure developed for the wild-type enzyme with a yield of about 30%. Analytical size exclusion chromatography showed that the mutant monoxygenase formed a tetramer, which is also the case for wild-type HbpA (14).

Major Reaction Products of Indole Hydroxylation by HbpA<sub>ind</sub>

To identify the major reaction products of indole hydroxylation, *in vitro* indigo formation assays were performed. After 30 min a sample was taken and immediately saturated with argon. The proteins were precipitated and separated by centrifugation. The pigments in the pellet were extracted with DMF and analyzed by TLC. They were identified as indigo and indirubin. Analysis of the aqueous phase by HPLC-MS revealed the presence of 3-hydroxyindole (indoxyl) and 2-indolinone (oxindole). When a sample was taken after 60 min assay time, isatin was also detected (Table I).

Substrate Spectrum of HbpA<sub>ind</sub>
To investigate the substrate range of the \textbf{HbpA\textsubscript{ind}} mutant monooxygenase, \textit{in vitro} assays were performed with 4-hydroxyindole, 5-hydroxyindole, and 5-bromoindole. For the variant enzyme color formation could be observed with all substrates while the control assays with the wild-type protein remained colorless. The pigment derived from 4-hydroxyindole was purple, that from 5-hydroxyindole orange, and that from 5-bromoindole was pink.

The polar reaction products of the assays with 4- and 5-hydroxyindole were analyzed with HPLC-MS. For this the \textit{in vitro} assay was stopped by the addition of perchloric acid and the proteins were spun down. Mass peaks (MH\textsuperscript{+}) at \textit{m/z} 150 were detected in the supernatants from both reactions. This mass correlates with the single hydroxylated substrates. The corresponding compounds eluted between 4.5 and 6.5 minutes when using 40\% (v/v) methanol as mobile phase. The main condensation products had a prominent molecular ion peak (MH\textsuperscript{+}) at \textit{m/z} 279 and had a retention time of 4.1 min for the assay with 4-hydroxyindole and of 3.5 min for the assay with the 5-substituted isomer. UV/Vis spectra showed the peak at 4.1 min to have a maximum at 494 nm, whereas the peak at 3.5 min had a maximum at 480 nm. Thus the molecular mass and the spectral properties indicate that the dihydroxy derivatives of indoxyl red were formed (Fig. 7).

\textbf{Catalytic Properties of HbpA and HbpA\textsubscript{ind}}

\textit{Specific Activities towards 2-Hydroxybiphenyl and Indole - In vitro} activity of the purified proteins towards the natural substrate 2-hydroxybiphenyl was determined by measuring substrate consumption and product formation with reverse phase HPLC. The \textit{k\textsubscript{cat}} of HbpA\textsubscript{ind} was significantly lower than that of the wild type enzyme (Table II). Indole hydroxylation activities were determined in assays with purified proteins and NADH regeneration by formate dehydrogenase from \textit{Candida boidinii}. The assay mix
containing the mutant monooxygenase showed a blue color within the first 30 min, whereas the assay mix with the wild type enzyme remained white. HbpA_\text{ind} formed up to 170 µM indigo, whereas hardly any indigo formation could be observed for HbpA (Fig. 4). The indole hydroxylation activity of HbpA_\text{ind} was approximately 20 mU mg\(^{-1}\) purified protein or about 18 times higher than the corresponding value for the wild type enzyme.

Equilibrium Binding of Substrates to HbpA and HbpA_\text{ind} - The affinities of the enzymes towards 2-hydroxybiphenyl and indole were determined by titration of the purified proteins with known concentrations of substrate (\textit{insets} Fig. 5). Plotting the absorption difference at a specific wavelength as a function of substrate concentration (Fig. 5) allowed the determination of the \(K_d\) values. Whereas the dissociation constants for 2-hydroxybiphenyl were in the same range for both proteins, the \(K_d\) value for indole was 20-fold lower for HbpA_\text{ind} than for HbpA (Table II).

Uncoupling of NADH Oxidation from 2-Hydroxybiphenyl Hydroxylation - Wild-type HbpA shows an uncoupling of NADH oxidation from 2-hydroxybiphenyl hydroxylation of 21% (18). Substitution of a single amino acid (Val368Ala in HbpA\(_{T1}\)) completely coupled these two reactions (18). In contrast, there was significant uncoupling of NADH oxidation from hydroxylation for HbpA_\text{ind}, compared to HbpA and HbpA\(_{T1}\) (Table III). To investigate whether the increased uncoupling in HbpA_\text{ind} is an effect of the combination of the two amino acid substitutions or only due to the D222V exchange, the single mutant D222V (HbpA\(_{D222V}\)) was constructed by site directed mutagenesis. Uncoupling of NADH oxidation from 2-hydroxybiphenyl hydroxylation was 3-fold higher for the HbpA\(_{D222V}\) mutant monooxygenase than for the wild-type protein (Table III).
DISCUSSION

**Hydroxylation of indole by HbpA<sub>ind</sub>**

Indole is oxidized by different oxygenases that contain either protein bound iron or cytochrome to activate molecular oxygen (6, 32). No flavoprotein oxygenase has thus far been shown to accept indole as a substrate. A 2-hydroxybiphenyl 3-monooxygenase variant (Hbp<sub>A<sub>ind</sub></sub>), which we obtained during directed evolution of HbpA, showed the ability to hydroxylate indole. The $k_{cat}/K_m$ of HbpA<sub>ind</sub> for indole was 330-fold higher than that of wild-type HbpA, and is in the same order as the catalytic efficiency determined for an engineered fatty-acid hydroxylase P450 BM-3 (33). This P450 variant was obtained by saturation mutagenesis and is the enzyme with the highest known catalytic efficiency towards indole.

Cultures of *E. coli* JM101 that synthesized HbpA<sub>ind</sub> during growth on LB medium had an indigo productivity of about 5 mg l<sup>-1</sup> h<sup>-1</sup>. In comparison, a recombinant *E. coli* HB101 culture that expressed the naphthalene dioxygenase genes produced about 1 mg l<sup>-1</sup> h<sup>-1</sup> during growth on the same medium to a similar cell density (2). *E. coli* cultures that synthesized human cytochrome P450s reached productivities of approximately 0.3 mg l<sup>-1</sup> h<sup>-1</sup> on fortified TB medium (6). Thus, the HbpA<sub>ind</sub> flavoprotein recombinant is considerably (5 to 15-fold) more active *in vivo* in the formation of indigo from complex medium. Analysis of the formed pigments also showed the presence of the byproduct indirubin, a structural isomer of indigo that is known to be formed by indole oxidizing enzymes (4, 10).

Indigo extracted from recombinant *E. coli* cell cultures showed only limited stability when stored in DMF at room temperature. This could be attributed to the pH of the solution. It is known from denim manufacturing, that at basic pH indigo is chemically
reduced to its water soluble form indigo white. In contrast, the formed indirubin was stable when extracted and stored in DMF.

**Major Reaction Products of Indole Hydroxylation**

The products of indole oxidation by different mono- and dioxygenases have been investigated. Indole-epoxide has been suggested as a product for the reaction catalyzed by styrene monooxygenase (7), and indoxyl (3-hydroxyindole) has been identified as a hydroxylation product of cytochrome P450 enzymes (6). Oxidation by naphthalene dioxygenase results in the formation of 2,3-dihydroxy-2,3-dihydroindole (2). All these reaction products are unstable and spontaneously form pigments. The identification of the intermediates formed during *in vitro* indigo assays with HbpA*ind* suggests a similar route as observed for the P450 enzymes (Fig. 6). The identification of 3-hydroxyindole and 2-indolinone indicates direct hydroxylation at the carbons of the pyrrole ring of indole. In contrast to the P450 enzymes, no hydroxylation at the benzene ring of the substrate was observed (6). The presence of isatin could have two origins: it was either produced by hydroxylation of indoxyl or oxindole, or by the decomposition of indigo and indirubin. The formation of indigo and indirubin from the enzymatic hydroxylation products of indole is spontaneous and biocatalyst independent. In short, condensation of two molecules of indoxyl followed by air oxidation leads to the production of indigo, while the condensation of indoxyl and 2-indolinone yields indirubin (28, 34). In addition, indirubin can also be formed by the reaction of indoxyl with isatin (4, 35). That this latter reaction does indeed take place in the case of indirubin formation by HbpA*ind* is supported by the fact that the ratio of indirubin to indigo increased with time when recombinant *E. coli* were grown on LB.

**Substrate spectrum of HbpA*ind***
Indirubin inhibits cyclin-dependent kinases and therefore belongs to a group of promising anticancer compounds (20). Analogs such as indirubin-3’-monoxime or halogenated indirubins show even higher potency (36). This increased biological activity can be attributed to the lower hydrophobicity of the derivatives compared to indirubin. Thus the uptake of the compound is facilitated and the probability that it reaches the biological sites of action is increased (37). In this context we investigated the substrate spectrum of HbpA\textsubscript{ind}. The variant showed activity with several indole derivatives, such as 4- and 5-hydroxyindole. The formed products are potentially interesting because their log$P$ values are significantly different from that of the unsubstituted indirubin and are hardly accessible by chemical means. However, analysis of the formed pigments showed that the dihydroxyindirubin derivatives were only a minor product of the reaction of HbpA\textsubscript{ind} with hydroxyindoles. The mass and the spectral properties of the major condensation product indicates that mainly the indoxyl red derivatives were formed. Indoxyl red is known to be formed from the reaction of 3-oxo 3$H$-indole with indole (38). The proposed pathway for the formation of the dihydroxy derivatives by HbpA\textsubscript{ind} from hydroxyindole is shown in Figure 7. The electron donating hydroxy-group at the benzene ring facilitates the oxidation of the indolinone compared to the unsubstituted compound and may explain why indoxyl red was not detected from the reaction of HbpA\textsubscript{ind} with indole. Indoxyl red shares a high degree of structural similarities with indirubin. The dihydroxy derivatives have a strongly decreased hydrophobicity and their potency in inhibiting cyclin-dependent kinases is worth investigating.

**Catalytic Properties of HbpA\textsubscript{ind}**

We characterized HbpA\textsubscript{ind} with respect to its catalytic properties. The variant activity towards indole was about 18-fold increased compared to the wild type enzyme. This
increase was concomitant with an enhanced affinity of the enzyme towards this substrate. The *in vitro* activity of the mutant monooxygenase towards the natural substrate 2-hydroxybiphenyl was significantly decreased, while its affinity towards this substrate remained unchanged. This is mostly due to a slower flavin reduction, as indicated by the reduced NADH oxidation rate. In addition, uncoupling of NADH oxidation from substrate hydroxylation was significantly increased in HbpA<sub>mut</sub>, HbpA<sub>mut</sub> evolved from the single mutant V368A (HbpA<sub>T1</sub>) by directed enzyme evolution. In contrast to wild-type HbpA, HbpA<sub>T1</sub> fully couples NADH oxidation to 2-hydroxybiphenyl hydroxylation. We have suggested that this is due to the stabilization and/or improved positioning of the flavin (C4a)-hydroperoxide towards the substrate (18). This enhanced hydroxylation efficiency was completely destroyed by the substitution D222V: in HbpA<sub>mut</sub> the uncoupling was twice that of the wild-type protein with 2-hydroxybiphenyl as substrate, while the unproductive NADH oxidation rate was similar in both enzymes. The single mutant HbpA<sub>D222V</sub> even showed a 3-fold increased uncoupling, which was concomitant with a 3-fold increased unproductive NADH oxidation rate. Thus, substitution D222V in HbpA<sub>mut</sub> and HbpA<sub>D222V</sub> directly increases the ratio of flavin (C4a)-hydroperoxide decay to 2,3-dihydroxybiphenyl formation (Fig. 8). This ratio is primarily influenced by the stabilization of the flavin (C4a)-hydroperoxide, the solvent access to the active site and the reactivity of the substrate towards electrophilic attack by the terminal oxygen of the peroxide. According to the structural model of HbpA (18), Asp<sup>222</sup> is located close to the bound substrate in the substrate-binding domain. Interestingly, Asp<sup>222</sup> of HbpA corresponds with Tyr<sup>201</sup> of *p*-hydroxybenzoate hydroxylase (PHBH) from *Pseudomonas fluorescens* (39, 40). In PHBH, Tyr<sup>201</sup> is critically involved in the ionization of the substrate, thus affecting the flavin movement and allowing efficient hydroxylation (41, 42). Substitution Tyr201Phe
results in an increased uncoupling of NADH oxidation from substrate hydroxylation up to 95% (43). In phenol 2-monoxygenase (PHHY) from *Trichosporon cutaneum*, Tyr^{289} is hydrogen bonded with the hydroxyl group of the substrate, comparable to Tyr^{201} in PHBH (44). Substitution of this residue by Phe increased the uncoupling from 10 to 34% (45). With respect to the localization of Asp^{222} in the HbpA model, the chemical properties of this residue, and the effects resulting from its substitution, it is likely that Asp^{222} plays a similar role in HbpA as do the tyrosine residues in PHBH and PHHY.

In summary, we characterized the HbpA<sub>ind</sub> mutant monooxygenase, the first flavoprotein able to hydroxylate indole. These investigations point to the importance of amino acid residue Asp^{222} in the catalytic cycle of HbpA. Thus, the results obtained here may serve as the basis for further elucidation of the mechanism of substrate activation in this enzyme.

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REFERENCES


FIGURE LEGENDS

Fig. 1: Scheme of in vitro indigo formation assay. HbpA, 2-hydroxybiphenyl 3-monoxygenase or HbpA\textsubscript{ind}; FDH, formate dehydrogenase.

Fig. 2: In vivo indigo formation by HbpA and HbpA\textsubscript{ind}. Formation of indigo during growth of recombinant \textit{E. coli} JM101 on LB broth synthesizing wild type HbpA or HbpA\textsubscript{ind}. Symbols for cell dry weight (CDW): □, wild-type HbpA; ○, HbpA\textsubscript{ind}. Symbols for indigo concentration: ■, wild-type HbpA; ●, HbpA\textsubscript{ind}.

Fig. 3: Indigo and indirubin. Indigo freshly extracted from recombinant \textit{E. coli} culture (right) and after incubation at room temperature for 72 hours (left).

Fig. 4: In vitro indigo formation by HbpA and HbpA\textsubscript{ind}. The assay contained 0.2 µM HbpA or HbpA\textsubscript{ind}, 0.25 U formate dehydrogenase, 160 mM sodium formate, 10 U catalase, 0.3 mM NADH, 2 mM indole in 1 ml 50 mM sodium phosphate buffer pH 7.5. Indigo formation was determined at 610 nm. ○, wild-type HbpA; ■, HbpA\textsubscript{ind}.

Fig. 5: Equilibrium binding of indole to wild-type HbpA and HbpA\textsubscript{ind}. A: Absorption changes at 490 nm during titration of 12 µM wild type HbpA with 0.3, 0.7, 1.3, 2.3, 4, and 6.7 mM indole. The inset shows the difference spectra in the presence of 0.3, 0.7, and 2.3 mM substrate. B: Absorption changes at 495 nm during titration of 12 µM HbpA\textsubscript{ind} with 33.3, 66.7, 100, 166.7, 266.7, 433.3, and 666.7 µM indole. The inset shows the difference spectra in the presence of 33.3, 66.7, and 100 µM substrate.
Fig. 6: Proposed pathway for the formation of indigo and indirubin by HbpA$_{ind}$. 1) indole; 2) 3-hydroxyindole (indoxyl), 2a) 3-indolinone; 3) 2-hydroxyindole, 3a) 2-indolinone (oxindole); 4) isatin; 5) indirubin; 6) indigo.

Fig. 7: Proposed pathway for the formation of dihydroxy derivatives of indoxyl red. 1) 4- or 5-hydroxyindole, 2) 4- or 5-hydroxyindoxyl 3) 4- or 5-hydroxy-2-indolinone 4) 4- or 5-hydroxy-3-oxo-3$H$-indole 5) 4,4'- or 5,5'-dihydroxyindoxyl red.

Fig. 8: Ratio of flavin (C4a)-hydroperoxide decay to 2,3-dihydroxybiphenyl formation in the catalytic cycle of HbpA and the variants. For each of the variants the rate of formation of the flavin (C4a)-hydroperoxide is normalized compared to wild-type enzyme, assuming flavin reduction to be the rate limiting step (15). EFlHOOH-S, flavin (C4a)-hydroperoxide enzyme-substrate complex; EFl$_{ox}$, enzyme containing oxidized flavin.
Table I:

**Major reaction products of indole hydroxylation by HbpA<sub>ind</sub>.** The major reaction products of *in vitro* indole assays were authenticated by reverse HPLC-MS. The assays contained 0.2 µM HbpA<sub>ind</sub>, 0.25 U formate dehydrogenase, 160 mM sodium formate, 10 U catalase, 0.3 mM NADH, and 2 mM indole in 1 ml 50 mM sodium phosphate buffer pH 7.5.

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<td>2-Indolinone</td>
<td>7.6</td>
<td><img src="image" alt="2-Indolinone" /></td>
<td>204, 249</td>
<td>134</td>
</tr>
<tr>
<td>(oxindole)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Indole</td>
<td>12.1</td>
<td><img src="image" alt="Indole" /></td>
<td>217, 270</td>
<td>118</td>
</tr>
</tbody>
</table>
* All compounds were compared to commercially obtained standards.
Table II:

$K_d$ and $k_{cat}$ values of wild-type HbpA and HbpA\_ind towards 2-hydroxybiphenyl and indole.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>2-Hydroxybiphenyl</th>
<th>Indole</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$k_{cat}$</td>
<td>$K_d$</td>
</tr>
<tr>
<td>HbpA</td>
<td>15.6</td>
<td>9.9±0.7</td>
</tr>
<tr>
<td>HbpA_ind</td>
<td>2.3</td>
<td>8.8±0.7</td>
</tr>
</tbody>
</table>

*a* Determined by measuring substrate consumption and product formation with reverse phase HPLC. Values are the average of 3 independent measurements and have a standard error <10%.

*b* Determined by *in vitro* indigo formation assay with NADH regeneration by formate dehydrogenase. Values are the average of 2 independent measurements and have a standard error <10%.

*c* Value adapted from Suske *et al.* (15).
Table III:

**Uncoupling of NADH oxidation from 2-hydroxybiphenyl hydroxylation**

The assays were performed at 30°C in 20 mM sodium phosphate buffer (pH 7.5) containing 0.3 mM NADH and 0.2 mM 2-hydroxybiphenyl.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>AA substitution</th>
<th>Specific activity</th>
<th>Unproductive NADH oxidation</th>
<th>Uncoupling</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>NADH oxidation$^a$</td>
<td>Product formation$^b$</td>
<td></td>
</tr>
<tr>
<td>HbpA$^c$</td>
<td>-</td>
<td>15.6 s$^{-1}$</td>
<td>12.3 s$^{-1}$</td>
<td>3.3 s$^{-1}$</td>
</tr>
<tr>
<td>HbpA$^c_{T1}$</td>
<td>V368A</td>
<td>16.2 s$^{-1}$</td>
<td>15.8 s$^{-1}$</td>
<td>0.4 s$^{-1}$</td>
</tr>
<tr>
<td>HbpA$^c_{D222V}$</td>
<td>D222V</td>
<td>15.0 s$^{-1}$</td>
<td>5.1 s$^{-1}$</td>
<td>9.9 s$^{-1}$</td>
</tr>
<tr>
<td>HbpA$^c_{ind}$</td>
<td>D222V/V368A</td>
<td>5.2 s$^{-1}$</td>
<td>2.3 s$^{-1}$</td>
<td>2.9 s$^{-1}$</td>
</tr>
</tbody>
</table>

$^a$ Determined by monitoring the NADH decrease at 340 nm.

$^b$ Determined by measuring substrate consumption and product formation with reverse phase HPLC.

$^c$ Values adapted from Meyer et al. (18).
Fig. 1:

\[
\begin{align*}
\text{O}_2 & \quad \text{H}_2\text{O} \\
\text{HbpA} & \quad \text{air oxidation} \\
\text{NADH} + \text{H}^+ & \quad \text{NAD}^+ \\
\text{FDH} & \quad \text{CO}_2 \quad \text{HCOOH}
\end{align*}
\]
Fig. 2:
Fig. 3:
Fig. 4:
Fig. 5:

A

B

Δ absorbance at 490 nm

Δ absorbance at 495 nm

wavelength [nm]

wavelength [nm]

indole [mM]

indole [µM]
Fig. 6:

Catalyzed by HbpA_{ind}:

- **3a** → **1**
- **1** → **2a**
- **3** → **2**
- **2** → **1**
- **2a** → **3a**

Spontaneous:

- **4** → **5**
- **4** → **6**
- **5** → **4**
- **6** → **4**

Condensation of:
- **2 + 3** or **2 + 4**
- **2 + 2**
Fig. 7:

1 + 4 → 5

1 → 2 → 3 → 4

HbpA_{ind} → oxidation
Fig. 8:

**HbpA**

\[
\begin{align*}
\text{100%} & \quad \xrightarrow{\text{2-hydroxyphenyl + H}_2\text{O}_2} \quad \xrightarrow{21:79} \quad \xrightarrow{\text{EFIIOH-S}} \quad \xrightarrow{\text{2,3-dihydroxyphenyl + H}_2\text{O}} \\
\end{align*}
\]

**HbpA_{T1}**

\[
\begin{align*}
\text{104%} & \quad \xrightarrow{\text{2-hydroxyphenyl + H}_2\text{O}_2} \quad \xrightarrow{3:97} \quad \xrightarrow{\text{EFIIOH-S}} \quad \xrightarrow{\text{2,3-dihydroxyphenyl + H}_2\text{O}} \\
\end{align*}
\]

**HbpA_{D222V}**

\[
\begin{align*}
\text{96%} & \quad \xrightarrow{\text{2-hydroxyphenyl + H}_2\text{O}_2} \quad \xrightarrow{66:34} \quad \xrightarrow{\text{EFIIOH-S}} \quad \xrightarrow{\text{2,3-dihydroxyphenyl + H}_2\text{O}} \\
\end{align*}
\]

**HbpA_{ind}**

\[
\begin{align*}
\text{33%} & \quad \xrightarrow{\text{2-hydroxyphenyl + H}_2\text{O}_2} \quad \xrightarrow{56:44} \quad \xrightarrow{\text{EFIIOH-S}} \quad \xrightarrow{\text{2,3-dihydroxyphenyl + H}_2\text{O}} \\
\end{align*}
\]