Hydroxylation of Indole by Laboratory-evolved 2-Hydroxybiphenyl 3-Monoxygenase*

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Directed enzyme evolution of 2-hydroxybiphenyl 3-monoxygenase (HbpA; EC 1.14.13.44) from Pseudomonas azelaica HBPI resulted in an enzyme variant (HbpA_{mut}) that hydroxylates indole and indole derivatives such as hydroxyindoles and 5-bromoindole. The wild-type protein does not catalyze these reactions. HbpA_{mut} contains amino acid substitutions D222V and V368A. The activity for indole hydroxylation was increased 18-fold in this variant. Concomitantly, the $K_d$ value for indole decreased from 1.5 mM to 78 $\mu$M. Investigation of the major reaction products of HbpA_{mut} with indole revealed hydroxylation at the carbons of the pyrrole ring of the substrate. Subsequent enzyme-independent condensation and oxidation of the reaction products led to the formation of indigo and indirubin. The activity of the HbpA_{mut} mutant monooxygenase for the natural substrate 2-hydroxybiphenyl was six times lower than that of the wild-type enzyme. In HbpA_{mut}, there was significantly increased uncoupling of NADH oxidation from 2-hydroxybiphenyl hydroxylation, which could be attributed to the substitution D222V. The position of Asp^{222} in HbpA, the chemical properties of this residue, and the effects of its substitution indicate that Asp^{222} is involved in substrate activation in HbpA.

Indole is produced from the aromatic amino acid tryptophan in tryptophanase-synthesizing bacteria such as Escherichia coli (1). Enzymes that oxidize 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-oxygenating enzymes was mainly aimed at the biotechnological production of indigo, we were especially interested in the formation of the by-product indirubin. Indirubin and its analogs have been identified as potent inhibitors of cyclin-dependent kinases (20). The crystal structure of cyclin-dependent kinase-2 in complex with indirubin derivatives showed that indirubin binds to the kinase 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).

**EXPERIMENTAL PROCEDURES**

**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- and 5-hydroxyindole were from ICN Pharmaceuticals (Cleveland, OH). All other chemicals were of the purest available quality and obtained from Fluka AG.

**Directed Evolution of HbpA**

Directed evolution of HbpA was performed 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 for the hydroxylation of indole formed deep blue colonies. The single mutant D222V (HbpA_{D222V}) was constructed using the QuickChange™ site-directed mutagenesis kit from Stratagene (La Jolla, CA).

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Determination of activity for 2-hydroxybiphenyl—The activity of recombinant E. coli JM101 for the formation of indigo was determined in JIM101 cells harboring a pUC18 derivative encoding HbpA or HbpAind. Symbols for indigo concentration are as follows: □, wild-type HbpA; and ○, HbpAind.

Protein Synthesis and Purification

Synthesis of wild-type HbpA and HbpAind was done in recombinant E. coli JM101 using M9 mineral medium and glycerol as the carbon source (17). After harvesting the cells, the proteins were purified according to the method described recently (18).

Analytical Methods

Determination of activity for 2-hydroxybiphenyl—The activity of wild-type HbpA and HbpAind was determined by measuring substrate consumption and product formation with reverse-phase high pressure liquid chromatography (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—The activity of recombinant E. coli JM101 for the formation of indigo was determined in 250-ml shaking flasks containing 50 ml of LB medium (22). Cultures of E. coli JM101 cells harboring a pUC18 derivative encoding HbpA or HbpAind were inoculated to A600 = 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 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 (ε610 = 15,900 liters mol⁻¹ cm⁻¹) (8).

Determination of in Vitro Indigo Formation—The activity for indole was determined using an assay with NADH regeneration by formate dehydrogenase from C. boidinii (Fig. 1) (26). The assay contained 0.2 μM HbpA or variant, 0.25 units of formate dehydrogenase, 160 mM sodium formate, 10 units of catechase from beef liver, 0.3 mM NADH, and 2 mM indole in 1 ml of 50 mM sodium phosphate buffer (pH 7.5). The assay was stopped by the addition of 20 μL of 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 μL purified wild-type HbpA and HbpAind were titrated with known concentrations of 2-hydroxybiphenyl or indole, and the resulting spectra were recorded using a Varian Cary E1 UV-visible spectrophotometer. Plotting absorbance at a specific wavelength allowed the calculation of the dissociation constants by weighted nonlinear regression analysis (Enzfitter, Elsevier-Biosoft, Cambridge, U. K.).

HPLC-Mass Spectroscopy (MS) Analysis—Analysis of compounds formed during in vitro indigo assays was done by reverse-phase HPLC-MS (Hewlett Packard 1100 MSD). The compounds were separated on a Hypersil ODS column (5 μm, 4.5 × 125 mm) and detected with a diode array detector and a mass spectrometer. Acidified (0.1% formic acid) H₂O (solvent A) and 50% methanol and 50% acetonitrile (solvent B) were applied as the mobile phase according to the following timetable: 0 to 8 min, 85:15 solvent A/solvent B, flow rate of 1 ml min⁻¹; gradient to 10 min, to 65:35 solvent A/solvent B, flow rate of 2 ml min⁻¹; to 15 min, 65:35 solvent A/solvent B, flow rate of 2 ml min⁻¹. Standards for isatin, 4- and 5-hydroxyindole, 2-indolinone, and indole were commercially available. 3-Indoxyl was prepared by dephosphorylating 3-indolyl phosphate with alkaline phosphatase 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 the mobile phase for the pigments derived from indole and with 40% (v/v) methanol for those derived from 4- and 5-hydroxyindole.

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 the mobile phase (28).

Electron Microscopy—For ultrathin sectioning, 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/Araldite (29). Sections cut from the Epon/Araldite preparation were contrasted with uranyl acetate and lead citrate.

Freeze-fracturing was carried out following standard procedures using a Balzers BAF 300 apparatus. 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-monoxygenase (HbpA) from \textit{P. azelaica} HBP1 by directed evolution (18). This work led to a mutant monoxygenase with increased activity for the hydroxylation of indole. The HbpA variant was denoted HbpA\textsubscript{ind}. \textit{E. coli} JM101 cultures synthesizing HbpA\textsubscript{ind} 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 DMF. It was authenticated as indigo by TLC with toluene/acetone (4:1) as the mobile phase and commercially available indigo as the standard. This analysis also revealed the presence of a major by-product. The \( R_p \) value of this red pigment corresponded to the \( R_p \) value determined earlier for indirubin (28). Analysis by HPLC-MS with 70\% (v/v) methanol as the mobile phase showed two prominent molecular ion (MH\(^+\)) peaks at \( m/z \) 283 with retention times of 3.7 and 4.2 min. The UV-visible 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 \textit{E. coli} JM101 cells growing on LB medium was quantified. Cultures expressing the hbpA\textsubscript{ind} gene accumulated 150 \( \mu \)M indigo within 8 h, whereas 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 \( \approx \)20\% of total cell protein.

Stability of Biotechnologically Produced Indigo

When pigments were extracted with DMF from recombinant \textit{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-visible 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) 10M hydrochloric acid stabilized the formed indigo, whereas the addition of 0.1\% (v/v) 10M sodium hydroxide or heating accelerated the disappearance of the blue color.

General Properties of HbpA\textsubscript{ind}

The mutant monoxygenase HbpA\textsubscript{ind} differs from wild-type HbpA by two amino acids: Asp\textsuperscript{222} was substituted by valine, and Val\textsuperscript{368} was substituted by alanine. HbpA\textsubscript{ind} was purified according to the procedure developed for the wild-type enzyme with a yield of \( \approx \)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\textsubscript{ind}

To identify the major reaction products of indole hydroxylation, \textit{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 of assay time, isatin was also detected (Table I).

| Compound | Retention time (min) | Structure | UV/Vis \( \lambda_{\text{max}} \) (nm) | MH\(^+\) |
|----------|---------------------|-----------|---------------------------------|---------|
| Isatin   | 5.5                 |           | 242, 302                        | 148     |
| 3-Hydroxyindole (indoxyl) | 6.1 | | 228, 381 | 134 |
| 2-Indolinone (oxindole) | 7.6 | | 204, 249 | 134 |
| Indole   | 12.1                | | 217, 270 | 118 |

\( ^{a} \text{All compounds were compared with commercially obtained standards.} \)

\( ^{b} \text{Determined by measuring substrate consumption and product formation by reverse-phase HPLC. Values are the average of three independent measurements and have a standard error <10\%.} \)

\( ^{c} \text{Determined by in vitro indigo formation assay with NADH regeneration by formate dehydrogenase. Values are the average of two independent measurements and have a standard error <10\%.} \)

\( ^{d} \text{Value adapted from Suske \textit{et al.} (15).} \)

| Enzyme | 2-Hydroxybiphenyl | Indole |
|--------|------------------|--------|
|        | \( k_{\text{cat}} \) \( \text{s}^{-1} \) | \( K_d \) \( \mu \text{M} \) | \( k_{\text{cat}}/K_d \) \( \text{s}^{-1} \mu \text{M}^{-1} \) | \( h_{\text{cat}} \) \( \text{s}^{-1} \) | \( K_d \) \( \mu \text{M} \) | \( h_{\text{cat}}/K_d \) \( \text{s}^{-1} \mu \text{M}^{-1} \) |
| HbpA   | 15.6             | 9.9 \( \pm \) 0.7    | 1.6 \( \times \) 10\(^6\) | 5 \( \times \) 10\(^{-3}\) | 1500 \( \pm \) 70 | 3.3 |
| HbpA\textsubscript{ind} | 2.3              | 8.8 \( \pm \) 0.7    | 2.6 \( \times \) 10\(^5\) | 9 \( \times \) 10\(^{-2}\) | 78 \( \pm \) 7  | 1.1 \( \times \) 10\(^2\) |

\( ^{a} \text{Determined by measuring substrate consumption and product formation by reverse-phase HPLC. Values are the average of three independent measurements and have a standard error <10\%.} \)

\( ^{b} \text{Determined by in vitro indigo formation assay with NADH regeneration by formate dehydrogenase. Values are the average of two independent measurements and have a standard error <10\%.} \)

\( ^{c} \text{Value adapted from Suske \textit{et al.} (15).} \)
that from 5-hydroxyindole was orange, and that from 5-bromoindole was pink.

The polar reaction products of the assays with 4- and 5-hydroxyindole were analyzed by HPLC-MS. For this, the in vitro assay was stopped by the addition of perchloric acid, and the proteins were spun down. Mass peaks (MH⁺) at 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 min when using 40% (v/v) methanol as the mobile phase. The main condensation products had a prominent molecular ion peak (MH⁺) at m/z 279 and had retention times of 4.1 min for the assay with 4-hydroxyindole and 3.5 min for the assay with the 5-substituted isomer. UV-visible 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 indicated that the dihydroxy derivatives of indoxyl red were formed (see Fig. 7).

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

**Specific Activities for 2-Hydroxybiphenyl and Indole**—The in vitro activity of the purified proteins for the natural substrate 2-hydroxybiphenyl was determined by measuring substrate consumption and product formation by reverse-phase HPLC. The 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 C. boidinii. The assay mixture containing the mutant monooxygenase showed a blue color within the first 30 min, whereas the assay mixture with the wild-type enzyme remained white. HbpA\textsubscript{ind} formed up to 170 \( \mu \text{M} \) indigo, whereas hardly any indigo formation could be observed for HbpA (Fig. 4). The indole hydroxylation activity of HbpA\textsubscript{ind} was \( \sim 20 \) milliunits mg\(^{-1}\) purified protein or \( \sim 18 \) times higher than the corresponding value for the wild-type enzyme.

**Equilibrium Binding of Substrates to HbpA and HbpA\textsubscript{ind}**—The affinities of the enzymes for 2-hydroxybiphenyl and indole were determined by titration of the purified proteins with known concentrations of substrate (Fig. 5, insets). Plotting the absorption difference at a specific wavelength as a function of substrate concentration (Fig. 5) allowed the determination of the K\textsubscript{d} values. Whereas the dissociation constants for 2-hydroxybiphenyl were in the same range for both proteins, the K\textsubscript{d} value for indole was 20-fold lower for HbpA\textsubscript{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 (V368A in HbpA\textsubscript{T1}) completely coupled these two reactions (18). In contrast, there was significant uncoupling of NADH oxidation from hydroxylation for HbpA\textsubscript{ind} compared with HbpA and HbpA\textsubscript{T1} (Table III). To investigate whether the increased uncoupling in HbpA\textsubscript{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\textsubscript{D222V}) was constructed by site-directed mutagenesis. Uncoupling of NADH oxidation from 2-hydroxybiphenyl hy-
droxylation was 3-fold higher for the HbpA<sub>D222V</sub> mutant monooxygenase than for the wild-type protein (Table III).

**DISCUSSION**

*Hydroxylation of Indole by Hbp<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>ind</sub>), which we obtained during directed evolution of HbpA, showed the ability to hydroxylate indole. The $k_{cat}/K_{d}$ of Hbp<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 the 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 for indole.

Cultures of *E. coli* JM101 cells that synthesized Hbp<sub>ind</sub> during growth on LB medium had an indigo productivity of $5 \text{ mg liter}^{-1} \text{ h}^{-1}$ on fortified terrific broth (TB) medium (6). Thus, the Hbp<sub>ind</sub> flavoprotein recombinant is considerably (5–15-fold) more active *in vivo* in the formation of indigo from complex medium. Analysis of the pigments formed also showed the presence of the by-product indirubin, a structural isomer of indigo that is known to be formed by indole-oxidizing enzymes (4, 10).

Indigo extracted from recombinant *E. coli* 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 of 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 human P450 cytochromes reached productivities of $0.3 \text{ mg liter}^{-1} \text{ h}^{-1}$ on fortified terrific broth (TB) medium (6). Thus, the Hbp<sub>ind</sub> flavoprotein recombinant is considerably (5–15-fold) more active *in vivo* in the formation of indigo from complex medium. Analysis of the pigments formed also showed the presence of the by-product indirubin, a structural isomer of indigo that is known to be formed by indole-oxidizing enzymes (4, 10).

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**TABLE III**

Uncoupling of NADH oxidation from 2-hydroxybiphenyl hydroxylation

| Enzyme | Amino acid substitution | NADH oxidation<sup>a</sup> | Product formation<sup>b</sup> | Unproductive NADH oxidation | Uncoupling<sup>c</sup> |
|--------|-------------------------|---------------------------|-----------------------------|----------------------------|---------------------|
| HbpA   |                         | 15.6                      | 12.3                        | 3.3                        | 21                  |
| HbpAT<sub>1</sub> V368A |                         | 16.2                      | 15.8                        | 0.4                        | 3                   |
| HbpAD<sub>222V</sub> | D222V                  | 15.0                      | 5.1                         | 9.9                        | 66                  |
| Hbp<sub>ind</sub> | D222V/V368A            | 5.2                       | 2.3                         | 2.9                        | 56                  |

<sup>a</sup> Determined by monitoring the NADH decrease at 340 nm.

<sup>b</sup> Determined by measuring substrate consumption and product formation by reverse-phase HPLC.

<sup>c</sup> Values adapted from Meyer *et al.* (18).

![Proposed pathway for the formation of indigo and indirubin by Hbp<sub>ind</sub>](image-url)
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<sub>ind</sub> 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 produced either 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, whereas 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<sub>ind</sub> is supported by the fact that the ratio of indirubin to indigo increased with time when recombinant E. coli cells were grown on LB medium.

Substrate Spectrum of HbpA<sub>ind</sub>—Indirubin inhibits cyclin-dependent kinases and therefore belongs to a group of promising anticancer compounds (20). Analogs such as indirubin 3’-monoxime and 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<sub>ind</sub>. The variant showed activity with several indole derivatives such as 4- and 5-hydroxyindole. The products of these reactions are potentially interesting because their log<sub>P</sub> 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<sub>ind</sub> with hydroxyindoles. The mass and the spectral properties of the major condensation product indicated that mainly the indoxyl red derivatives were formed. Indoxyl red is known to be formed from the reaction of 3-oxo-3H-indole with indole (38). The proposed pathway for the formation of the dihydroxy derivatives by HbpA<sub>ind</sub> from hydroxyindoles is shown in Fig. 7. The electron-donating hydroxyl group at the benzene ring facilitates the oxidation of the indolinone compared with the unsubstituted compound and may explain why indoxyl red was not detected from the reaction of HbpA<sub>ind</sub> 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<sub>ind</sub>—We characterized HbpA<sub>ind</sub> with respect to its catalytic properties. The variant’s activity for indole was increased by 18-fold compared with the wild-type enzyme. This increase was concomitant with an enhanced affinity of the enzyme for this substrate. The in vitro activity of the mutant monooxygenase for the natural substrate 2-hydroxybiphenyl was significantly decreased, whereas its affinity for this substrate remained unchanged. This is mostly due to a slower flavin reduction, as indicated by the reduced NADH.
In summary, we characterized the HbpA<sub>nd</sub> mutant monooxygenase, the first flavoprotein able to hydroxylate indole. These investigations point to the importance of Asp<sup>222</sup> 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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