Catalytic Mechanism of 2-Hydroxybiphenyl 3-Monoxygenase, a Flavoprotein from *Pseudomonas azelaica* HBP1*

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2-Hydroxybiphenyl 3-monoxygenase (EC 1.14.13.44) from *Pseudomonas azelaica* HBP1 is an FAD-dependent aromatic hydroxylase that catalyzes the conversion of 2-hydroxybiphenyl to 2,3-dihydroxybiphenyl in the presence of NADH and oxygen. The catalytic mechanism of this three-substrate reaction was investigated at 7 °C by stopped-flow absorption spectroscopy. Various individual steps associated with catalysis were readily observed at pH 7.5, the optimum pH for enzyme turnover. Anaerobic reduction of the free enzyme by NADH is a biphasic process, most likely reflecting the presence of two distinct enzyme forms. Binding of 2-hydroxybiphenyl stimulated the rate of enzyme reduction by NADH by 2 orders of magnitude. The anaerobic reduction of the enzyme-substrate complex involved the formation of a transient charge-transfer complex between the reduced flavin and NAD. A similar transient intermediate was formed when the enzyme was complexed with the substrate analog 2-sec-butylphenol or with the non-substrate effector 2,3-dihydroxybiphenyl. Excess NAD strongly stabilized the charge-transfer complexes but did not give rise to the appearance of any intermediate during the reduction of uncomplexed enzyme. Free reduced 2-hydroxybiphenyl 3-monoxygenase reacted rapidly with oxygen to form oxidized enzyme with no appearance of intermediates during this reaction. In the presence of 2-hydroxybiphenyl, two consecutive spectral intermediates were observed which were assigned to the flavin C(4a)-hydroperoxide and the flavin C(4a)-hydroxide, respectively. No oxygenated flavin intermediates were observed when the enzyme was in complex with 2,3-dihydroxybiphenyl. Monovalent anions retarded the dehydration of the flavin C(4a)-hydroxide without stabilization of additional intermediates. The kinetic data for 2-hydroxybiphenyl 3-monoxygenase are consistent with a ternary complex mechanism in which the aromatic substrate has strict control in both the reductive and oxidative half-reaction in a way that reactions leading to substrate hydroxylation are favored over those leading to the futile formation of hydroperoxide. NAD− release from the reduced enzyme-substrate complex is the slowest step in catalysis.

2-Hydroxybiphenyl 3-monoxygenase (EC 1.14.13.44) is an inducible flavoenzyme involved in the degradation of the fungicide 2-hydroxybiphenyl by the soil bacterium *Pseudomonas azelaica* HBP1 (1, 2). The microbial degradation of 2-hydroxybiphenyl proceeds through an oxidative meta-cleavage pathway. 2-Hydroxybiphenyl 3-monoxygenase catalyzes the first step of this pathway, i.e. the ortho-hydroxylation of 2-hydroxybiphenyl to 2,3-dihydroxybiphenyl in the presence of NADH and oxygen as shown in Scheme 1.

2-Hydroxybiphenyl 3-monoxygenase is a homotetramer, and each 60-kDa subunit contains a noncovalently bound FAD (2). The enzyme has a unique substrate specificity as it hydroxylates also 2,2′-dihydroxybiphenyl, 2,5-dihydroxybiphenyl, and a number of 2-alkylphenols to the corresponding 3-substituted catechols (3, 4). Because of its catalytic properties the enzyme has successfully been applied as a biocatalyst for the production of various 3-substituted catechols on the gram scale (5). The physiological product 2,3-dihydroxybiphenyl is a non-substrate effector for 2-hydroxybiphenyl 3-monoxygenase stimulating the nonproductive oxidation of NADH leading to H2O2 (4). Partial uncoupling of flavin reduction from hydroxylation by certain effectors and even the reaction product is a rather common feature among flavoprotein aromatic hydroxylases (6–9).

The gene encoding 2-hydroxybiphenyl 3-monoxygenase from *P. azelaica* HBP1 has recently been cloned and overexpressed in *Escherichia coli* (4). Sequence alignments show that 2-hydroxybiphenyl 3-monoxygenase belongs to the family of flavoprotein hydroxylases (2). Besides two fingerprints for the FAD binding, these enzymes share a conserved sequence motif with a putative dual function in FAD/NAD(P)H binding (10).

The catalytic mechanism of flavoprotein aromatic hydroxylases has extensively been studied by rapid-reaction techniques. The reaction cycle (Scheme 2) that was developed for *p*-hydroxybenzoate hydroxylase (11–13) also holds for a wide range of other flavoprotein aromatic hydroxylases (14–21). One of the most characteristic features of the flavoprotein aromatic hydroxylases is the control function of the aromatic substrate over the reduction of the flavin by NAD(P)H (6, 22, 23). Once a ternary complex of the enzyme-bound flavin with substrate and NAD(P)H has formed either in a random or in an ordered sequential manner, the enzyme-bound flavin is readily reduced by NAD(P)H within this complex. The reduced enzyme in complex with substrate and NAD(P)H− is kinetically evident from a transient long wavelength absorbance characteristic of a charge-transfer complex (21, 23). Release of NAD(P)H leads to a decay of the charge-transfer complex and completes the reductive half-reaction. The reduced enzyme-substrate complex subsequently reacts with oxygen in a second-order reaction yielding the flavin C(4a)-hydroperoxide, often referred to as intermediate I (11, 24). The binding of the substrate is essential to stabilize this oxygenated flavin species, which, in the absence

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of substrate, instantly decays to hydrogen peroxide and the oxidized enzyme. The distal oxygen of the flavin C(4a)-hydroperoxide is assumed to attack the substrate carbon ortho or para to the existing hydroxyl group by an electrophilic mechanism. Concomitant with substrate hydroxylation, the formation of the so-called intermediate II can be observed. This intermediate is seen only with the existing hydroxyl group by an electrophilic mechanism.

Our studies were aimed at elucidating the catalytic mechanism of 2-hydroxybiphenyl 3-monoxygenase. To that end, we investigated the influence of various enzyme ligands on individual steps of the oxidative half-reaction. The kinetic properties of 2-hydroxybiphenyl 3-monoxygenase were investigated by steady-state and kinetic experiments performed in air-saturated 50 mM HEPES buffer (pH 7.5). We then measured the formation of flavin charge-transfer complexes (23, 29). To ensure anaerobic conditions, 10 mM glucose and catalytic amounts of glucose oxidase were added to argon-flushed buffer and enzyme solutions. Moreover, the reaction syringes were immersed in a thermostated water bath where nitrogen was continuously bubbled to avoid contamination with oxygen through Teflon valves and stoppers.

Enzyme Purification—2-Hydroxybiphenyl 3-monoxygenase was purified from Escherichia coli JM101 harboring the hhpA gene, which encodes 2-hydroxybiphenyl 3-monoxygenase from P. azelaiaca HBP1, as reported earlier (2). The purified enzyme was stored at a concentration of 23 mg ml⁻¹ in 20 mM potassium phosphate buffer (pH 7.0) at −70 °C. Prior to use, an appropriate amount of enzyme was incubated with an FAD solution (1 mM). Excess FAD was removed by means of size exclusion chromatography on a Bio-Gel P-6DG column (Bio-Rad).

MATERIALS AND METHODS

Analytical Methods—Dissociation constants of enzyme-ligand complexes were determined spectrophotometrically with an Amino SP-500c spectrophotometer as described elsewhere (28). 2-Hydroxybiphenyl 3-monoxygenase activity was routinely assayed with an Hewlett-Packard HP8453 diode array spectrophotometer (Hewlett-Packard, Palo Alto, CA) at 30 °C. The assay mixture contained air-saturated 50 m M HEPES buffer (pH 7.5), 0.1 mM 2-hydroxybiphenyl, and 0.15 mM NADH. Buffers used for binding studies and kinetic experiments were 50 m M MES (pH 6.0–6.6), 50 mM HEPES (pH 7.0–8.0), and 50 mM TAPS (pH 8.5–9.0).

Steady-state kinetics were performed in air-saturated 50 mM HEPES buffer (pH 7.5), using a DW-2000 double beam spectrophotometer. The instrument was equipped with thermostated cell holders and interfaced to a microcomputer for data acquisition and data handling.

Stopped-flow Kinetics—Stopped-flow kinetic traces were recorded at 7 or 30 °C by means of a Hi-Tech SF-51 stopped-flow apparatus (Hi-Tech Scientific, Salisbury, UK) with a dead time of 1.3 ms. Spectral scans were recorded with a Hi-Tech M300 monochromator diode array detector (Hi-Tech Scientific, Salisbury, UK) that had a dead time of 5.6 ms. A total number of 96 scans could be collected in each single experiment with a minimum length of 0.96 s. Deconvolution analysis of spectral data was done with the Specfit Global Analysis program, version 2.10 (Spectrum Software Association, Chapel Hill, NC). Concentration values in stopped-flow experiments always refer to the final concentration obtained after mixing equal volumes of enzyme and reagents.

The anaerobic reduction of protein-bound FAD was monitored at 450 nm either in the absence or in the presence of saturating amounts of substrate, effector, and varying concentrations of NADH. Experiments were performed in 50 mM HEPES buffer (pH 7.5) containing 1 mM EDTA. Second-order rate constants and dissociation constants were determined by non-linear curve-fitting routines. The kinetics of enzyme reduction were also measured at longer wavelengths (>600 nm) in order to detect the formation of flavin charge-transfer complexes (23, 29).

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Table I

Dissociation constants of complexes between 2-hydroxybiphenyl 3-monooxygenase and aromatic ligands as determined by fluorimetric and spectral perturbation titration experiments

| Substrate                | pH   | Dissociation constant | Fluorescence spectroscopy | Absorption spectroscopy |
|--------------------------|------|-----------------------|---------------------------|-------------------------|
| 2-Hydroxybiphenyl        | 6.0  | 10.0 ± 0.6            | 16.5 ± 2.0                |                         |
|                          | 7.5  | 6.2 ± 0.4             | 9.9 ± 0.7                 |                         |
|                          | 9.0  | ND                    | 9.9 ± 0.8                 |                         |
| 2-sec-Butylphenol        | 6.0  | 22.3 ± 1.4            | ND                        |                         |
| 2-Propylphenol           | 6.0  | 15.3 ± 1.0            | 21.0 ± 2.8                |                         |
|                          | 7.5  | 30.4 ± 2.0            | ND                        |                         |
| 2,3-Dihydroxybiphenyl    | 6.0  | 112.4 ± 5.8           | ND                        |                         |
|                          | 7.5  | 37.4 ± 2.4            | 60.0 ± 5.0                |                         |

*ND, not determined.

Fig. 1. Spectral properties of 2-hydroxybiphenyl 3-monooxygenase. The absorption spectra were recorded in 50 mM HEPES at a pH of 7.5. A, optical spectra of a 6 μM solution of free enzyme before (spectrum 1) and after incubation (10 min) with 0.1% SDS (spectrum 2). B, difference spectra of a 20 μM solution of free enzyme as a reference and the same solution containing 2.5, 5.0, 9.8, 14.6, 19.2, 28.6, 37.7, 46.7, and 89.3 μM 2-hydroxybiphenyl. The inset shows a plot of the absorption changes at 502 nm as a function of the concentration of 2-hydroxybiphenyl.

(spectrum 1). Although rather unusual among flavoenzymes, a similar long wavelength absorbance was observed for melilotate hydroxylase (30) and pentachlorophenol hydroxylase (31). From the absorbance difference between free and protein-bound flavin (Fig. 1A), a value of 9.7 μs⁻¹ cm⁻¹ was estimated for the molar absorption coefficient (ε₄₅₀) of 2-hydroxybiphenyl 3-monooxygenase at a pH of 7.5. This value is in the same range as that reported for 4-p-hydroxybenzoate hydroxylase (28, 32).

Equilibrium Binding of Aromatic Ligands to 2-Hydroxybiphenyl 3-Monooxygenase—Previous results from steady-state kinetics revealed that 2-hydroxybiphenyl 3-monooxygenase has a rather low Michaelis constant (Kₘ) for 2-hydroxybiphenyl (apparent Kₘ = 2.8 μM), a fact which indicates a high affinity toward the aromatic substrate (2). In the present study, the interaction between the oxidized enzyme and aromatic ligands was investigated by static titration experiments. Binding of 2-hydroxybiphenyl led to marked perturbations in the absorbance of protein-bound FAD. The flavin atypical absorbance at wavelengths higher than 520 nm (cf. Fig. 1A, spectrum 1) disappeared upon binding of 2-hydroxybiphenyl. A possible explanation is that binding of 2-hydroxybiphenyl disrupts a charge-transfer interaction between the oxidized flavin and an active site residue. Fig. 1B shows the absorption difference spectra recorded upon titration of 2-hydroxybiphenyl 3-monooxygenase with 2-hydroxybiphenyl. From plotting the absorption changes at 502 nm as a function of substrate concentration and assuming simple 1:1 binding (inset of Fig. 1B), a dissociation constant for the enzyme-substrate complex of about 10 μM was estimated. As can be seen from Table I, the dissociation constant of the enzyme-2-hydroxybiphenyl complex did not strongly vary between pH 6 and pH 9.

Qualitatively similar absorption difference spectra as shown in Fig. 1B were observed when the oxidized enzyme was titrated with 2-sec-butylphenol. From treating the data according to simple 1:1 binding, a dissociation constant for the enzyme-2-sec-butylphenol of about 21 μM was estimated (Table I). Titration of 2-hydroxybiphenyl 3-monooxygenase with 2,3-dihydroxybiphenyl led to somewhat different spectral perturbations (not shown). Again, the long wavelength absorbance above 500 nm disappeared, but the increase in absorbance around 370 and 440 nm (cf. Fig. 1B) was far less intense than with 2-hydroxybiphenyl. Nevertheless, stoichiometric binding was observed, yielding a dissociation constant for the enzyme-2,3-dihydroxybiphenyl complex of about 60 μM (Table I).

Upon binding of FAD to the apoprotein, the flavin fluorescence is usually quenched. With 2-hydroxybiphenyl 3-monooxygenase, the relative fluorescence quantum yield of protein-bound FAD was about 1% that of free FAD. Since the flavin fluorescence emission at 535 nm of 2-hydroxybiphenyl 3-monooxygenase showed marked changes upon the binding of aromatic ligands, this feature was used to determine dissociation constants of the enzyme-ligand complexes. Binding of 2-hydroxybiphenyl to 2-hydroxybiphenyl 3-monooxygenase at pH 7.5 resulted in a 2-fold increase in the fluorescence of protein-bound FAD. The binding data could be described by simple 1:1 binding. A dissociation constant for the oxidized enzyme-2-hydroxybiphenyl complex of about 6 μM was calculated from the data (Table I). This value is in agreement with the value of the dissociation constant derived from absorption spectroscopy. Similar fluorescence titration experiments revealed that 2-hydroxybiphenyl 3-monooxygenase tightly interacts with the substrate analogs 2-sec-butylphenol and 2-propylphenol (Table I). However, a somewhat weaker binding, especially at a pH of 6.0, was observed for the non-substrate effector 2,3-dihydroxybiphenyl (Table I).
**Steady-state Kinetics**—The specific activity of 2-hydroxybiphenyl 3-monoxygenase in 50 mM HEPES (pH 7.5) at 30 °C was 5.8 ± 0.2 units mg⁻¹. This value, based on flavin content analysis and a subunit molecular mass of 63.8 kDa (10), is considerably higher than the value originally derived from colorimetric protein determinations (2). The activation energy of the overall catalytic reaction of 2-hydroxybiphenyl 3-monoxygenase was determined using the standard assay procedure at pH 7.5. A plot of \( \ln k \) versus \( 1/T \) in the range 7–30 °C yielded a straight line, from which an activation energy of 49.2 ± 0.3 kJ mol⁻¹ was estimated (not shown). At 7 °C, an apparent \( V_{\text{max}} \) value of 1.3 ± 0.1 units mg⁻¹ and apparent \( K_{\text{m}} \) values, \( K_{\text{m,NADH}} \) NADH = 9.7 ± 1.8 \( \mu \)M and \( K_{\text{m,2-hydroxybiphenyl}} \) = 1.9 ± 0.4 \( \mu \)M, were estimated. The \( V_{\text{max}} \) at 7 °C corresponds to a turnover rate, \( k_{\text{cat}} \) = 1.4 ± 0.1 s⁻¹. It should be noted here that this value for the turnover rate refers to the enzyme monomer and not to the tetramer as was done in Ref. 2. No effort was made to determine the Michaelis constant for oxygen at 7 °C. However, from earlier kinetic data (2) and the high solubility of oxygen at low temperature, it is assumed that the estimated turnover rate approaches maximal rate conditions.

**Reduction of 2-Hydroxybiphenyl 3-Monoxygenase by NADH**—The mechanism of interaction between 2-hydroxybiphenyl 3-monoxygenase and NADH was studied at pH 7.5 (30 °C) by stopped-flow absorption spectroscopy. Anaerobic reduction of uncomplexed 2-hydroxybiphenyl 3-monoxygenase by NADH (monitored at 450 nm) was rather slow, taking seconds to complete (Fig. 2A). No transient absorbance changes above 500 nm, which would indicate the formation of flavin semiquinones or charge-transfer complexes, were observed. Reduction of the free enzyme by NADH was clearly not a monophasic process. Fitting the data to a two-exponential function showed that the reaction was biphasic. The amplitudes of the two phases remained constant at a ratio of 50:50 when the reaction was performed with NADH at concentrations between 25 \( \mu \)M and 5 mM. A possible explanation for the biphasic kinetics is the presence of two distinct enzyme populations in the absence of substrate (15, 33). From treating the data according to such a model, a maximum first-order rate constant at an infinite NADH concentration of 5.5 s⁻¹ and a dissociation constant, \( K_d \), NADH = 5.8 mM, were estimated for the fast process, whereas a maximum rate constant at infinite NADH concentration of 1.8 s⁻¹ and a dissociation constant, \( K_d \), NADH = 7.3 mM NADH, were calculated for the slow process (inset Fig. 2A, Table II). The values for the reduction rate of the free enzyme are similar to those for the turnover rate of the NADH oxidase activity of 2-hydroxybiphenyl 3-monoxygenase (2), which confirms that flavin reduction is rate-limiting in this process.

**Reduction of Enzyme-Ligand Complexes by NADH**—The substrate 2-hydroxybiphenyl acts as an effector and strongly stimulates the anaerobic reduction of 2-hydroxybiphenyl 3-monoxygenase by NADH. The rate of enzyme reduction as determined from the decrease in absorbance at 450 nm was dependent on the NADH concentration, the reaction being monophasic at all NADH concentrations studied (Fig. 2B). The maximum first-order rate constant at an infinite NADH concentration was 262 s⁻¹, with a dissociation constant of NADH from the enzyme-2-hydroxybiphenyl complex of 1.6 mM. This value of the dissociation constant is lower than that estimated for the free enzyme, which suggests that binding of NADH to the enzyme-substrate complex is preferred. 2-sec-Butylphenol, another efficient substrate of 2-hydroxybiphenyl 3-monoxygenase (2), was also tested for its effector properties. Anaerobic reduction of the enzyme-2-sec-butylphenol complex was significantly slower than of the enzyme-2-hydroxybiphenyl complex and was not monophasic. Similar to the free enzyme, biphasic reduction kinetics were observed. The two amplitudes obtained by fitting the data to a two-exponential function revealed an equal contribution of both processes which did not vary with the concentration of NADH between 50 \( \mu \)M and 2 mM (data not shown). Assuming two enzyme populations, we calculated a maximum reduction rate of 137 s⁻¹ and a dissociation constant, \( K_d \) NADH = 1.8 mM, for the fast process, and a maximum reduction rate of 36 s⁻¹ and a dissociation constant, \( K_d \) NADH = 3.6 mM, for the slow process (Table II). These data show that 2-sec-butylphenol is a strong effector that highly stimulates the reduction of protein-
Kinetic parameters for the reductive half-reaction of 2-hydroxybiphenyl 3-monoxygenase

The anaerobic reduction experiments were performed at 30 °C in 50 mM HEPES at a pH of 7.5. For experimental details see Figs. 2 and 3. Kd, NADH is the dissociation constant of NADH from the free enzyme or enzyme-ligand complexes. k2 (cf. Scheme 3) is the maximal first-order rate constant for the reduction of the enzyme at an infinite concentration of NADH.

| Ligand-enzyme complex          | k2  | Kd  |
|-------------------------------|-----|-----|
| Free enzyme                   | 5.8 | 5.5 |
| 2-Hydroxybiphenyl-enzyme complex | 1.6 | 262 |
| 2-sec-Butylphenol-enzyme complex | 1.8 | 137 |
| 2,3-Dihydroxybiphenyl-enzyme complex | 3.6 | 36  |
|                               | 8.6 | 163 |
|                               | 6.8 | 26  |

*Assuming two enzyme species with different reduction kinetics.

Again, biphasic kinetics were observed when the NADH-dependent anaerobic reduction of the enzyme-2,3-dihydroxybiphenyl complex was monitored in the stopped-flow apparatus. The extrapolated rate constants and the ratio of the amplitudes for both processes were in the same range as observed for the enzyme-2-sec-butylphenol complex. However, the estimated dissociation constants of NADH were relatively high (Table II), which suggests that the interaction between NADH and the enzyme-product complex is rather weak.

**Formation of Charge Transfer Intermediates**—To gain more insight in the sequence of events during the reductive half-reaction of 2-hydroxybiphenyl 3-monoxygenase, we studied the reduction of the enzyme-2-hydroxybiphenyl complex by NADH in further detail. Monitoring the reduction reaction at wavelengths above 500 nm, we observed small absorbance changes that are characteristic of the transient stabilization of charge-transfer complexes between protein-bound flavin and NADPH (23). Fig. 3A shows typical reaction traces recorded at 450, 550, and 700 nm. From the rise and fall traces at 550 and 700 nm, it is evident that at least one transient intermediate was formed. Based on the absorption coefficient at 452 nm (ε452) of 9.7 M⁻¹ cm⁻¹ for the oxidized enzyme, an absorption coefficient of 1.1 M⁻¹ cm⁻¹ for the intermediate was calculated from the maximal absorbance at 700 nm. The rate constant for the formation of the charge transfer intermediate corresponded perfectly to the observed flavin reduction rate (k2 = 139.6 s⁻¹) measured at 450 nm (Table III). This finding corroborates that flavin reduction and charge transfer complex formation actually represent the same reaction step. The presumed reaction sequence for the reductive half-reaction is shown in Scheme 3.

\[
\begin{align*}
EFlS + NADH & \rightarrow EFlS-NADH \rightarrow EFlS^+ \\
-\text{NAD}^+ & \rightarrow EFlS^+ + \text{NAD}^+
\end{align*}
\]

**Scheme 3.** Reductive half-reaction for 2-hydroxybiphenyl 3-monoxygenase complexed with 2-hydroxybiphenyl.

where EFlS indicates oxidized enzyme-substrate complex; EFlS-NADH and EFlredS-NAD⁺ indicate charge transfer complexes; and EFlredS indicates reduced enzyme-substrate complex.

Since the rate constant for the formation of the intermediate (k2) was much faster than its decay (k3), we conclude that only EFlredS-NAD⁺ (ε700 = 1.1 M⁻¹ cm⁻¹) contributes to the absorbance of the charge transfer complex. Furthermore, these data suggest that the release of NAD⁺ from the reduced enzyme-substrate complex is the slowest step during the reductive half-reaction (Table III).

At 7 °C, the reduction of the enzyme-2-hydroxybiphenyl complex by NADH was approximately five times slower than at 30 °C. The rate constants for the formation of the charge-transfer complex determined at 550 and 700 nm agreed again well with the rate constant found for the reduction of protein-bound FAD at 450 nm (k2, Table III). Interestingly, the decay of the charge-transfer complex at 7 °C was approximately 1910 ms with intervals of 20 ms. Spectra A-C are the spectra of the oxidized enzyme, the intermediate, and of the reduced enzyme, respectively. The data were fitted to a model with two consecutive reactions (A → B → C). The inset shows the simulated concentrations of the three components during the anaerobic reduction by NADH.

**Fig. 3.** Time course of absorbance upon reduction of the enzyme-substrate complex by NADH. The experiments were performed at 7 °C in the stopped-flow spectrophotometer. Both syringes contained 0.2 mM 2-hydroxybiphenyl in 50 mM HEPES, 1 mM EDTA, at pH of 7.5. A, anaerobic reduction of a solution of 21 μM enzyme in complex with 2-hydroxybiphenyl by 2 mM NADH. Original spectral scans (not shown) were recorded from 5.6 to 1910 ms with intervals of 20 ms. Spectra A-C are the spectra of the oxidized enzyme, the intermediate, and of the reduced enzyme, respectively. The data were fitted to a model with two consecutive reactions (A → B → C). The inset shows the simulated concentrations of the three components during the anaerobic reduction by NADH.
The experiments were performed in 50 mM HEPES at a pH of 7.5. For experimental details, see Fig. 3. Symbols: T, temperature; λ, wavelength; k$_{\text{f}}$, and k$_{\text{a}}$, apparent first-order rate constants for the formation and the decay of the charge-transfer complex (cf. Scheme 3).

| T (°C) | λ (nm) | k$_{\text{f}}$ (s$^{-1}$) | k$_{\text{a}}$ (s$^{-1}$) |
|-------|-------|----------------|----------------|
| 30    | 450   | 139.6          |                 |
| 7     | 450   | 28.6           | 11.1           |
| 7     | 550   | 22.5           | 1.1            |
| 7     | 700   | 30.2           | 0.9            |

*Data were obtained by deconvolution of spectral data from diode array detection experiments (cf. Fig. 3B).

oxidized flavin, reduced flavin, and the transient intermediate. These spectra were obtained by deconvolution analysis of the original spectral data. The inset of Fig. 3B shows the simulated concentrations of the three spectral species during the reaction. The rate constant for the irreversible transition from the oxidized flavin to the charge transfer intermediate (k$_{\text{f}}$ in Scheme 3) was similar to that calculated from single wavelength scans at 550 and 700 nm (Table III). Moreover, the calculated decay rate (k$_{\text{a}}$ in Scheme 3) of 1.0 s$^{-1}$ was in good agreement with the rate constants determined from single scan data. The transient formation of a charge-transfer complex was also observed during reduction of the enzyme-2-sec-butylphenol complex and the enzyme-2,3-dihydroxybiphenyl complex. For all these enzyme-ligand complexes, the rate of NAD$^+$ dissociation was the slowest step in the reductive half-reaction (results not shown).

**Reduction of the Enzyme-Ligand Complexes in the Presence of NAD$^+$**—To get a deeper insight into the nature of the biphasic behavior observed upon mixing free enzyme or several enzyme-ligand complexes with NADH, we studied the anaerobic reduction in the presence of excess NAD$^+$. If the biphasic kinetics were the result of two enzyme conformations at equilibrium each having different reduction kinetics, preincubation of the enzyme with NAD$^+$ would eliminate one of the two phases simply by alteration of the equilibrium (33). However, oxidized 2-hydroxybiphenyl 3-monoxygenase preincubated with 5 mM NAD$^+$ retained the typical biphasic reduction kinetics, both at 7 and 30 °C (Table IV). Since the presence of an excess of NAD$^+$ reduced the apparent reduction rates only slightly and did not hinder the reduction reaction per se, the binding of NAD$^+$ to the oxidized 2-hydroxybiphenyl 3-monoxygenase was considered to be rather weak.

In another series of experiments we investigated the influence of excess NAD$^+$ on the reduction of the enzyme-product complex by NADH at 7 °C. First of all, the reduction of the enzyme-2,3-dihydroxybiphenyl complex was followed in the absence of NAD$^+$. The transient formation of a charge-transfer complex can easily be seen from Fig. 4A. A completely different picture was obtained when the same reaction was analyzed in the presence of 5 mM NAD$^+$ (Fig. 4B). The reaction trace recorded at 700 nm reflects the formation of a stable charge-transfer complex. The fact that this complex did not decay when NAD$^+$ was present in excess was taken as a proof that the observed intermediate was in fact similar to the EFL$_{\text{red}}$-S-NAD$^+$ species shown in Scheme 3. Fitting of the data to a two-exponential function revealed that the reaction traces recorded at 700 nm were biphasic, the reaction rates (k$_{\text{f}}$) being 4.6 s$^{-1}$ for the faster rate and 0.7 s$^{-1}$ for the slower rate. In contrast, the reaction traces recorded at 450 nm were best fit to a model based on a three-exponential function that suggests that more than two first-order reactions simultaneously contributed to the process (Table IV).

Diode array detection was used to study the anaerobic reduction of free enzyme, the enzyme-substrate complex, and the enzyme-product complex in the presence of excess NAD$^+$ at 7°C in more detail. As expected, no intermediate formation occurred during reduction of the free enzyme. For the reduction of the enzyme-substrate complex, the rate constants for the formation and the decay of the charge-transfer complex were 19 s$^{-1}$ (k$_{\text{f}}$) and 0.009 s$^{-1}$ (k$_{\text{a}}$), respectively. For comparison, the corresponding rate constants for the enzyme-product complex determined from deconvolution of spectral data were 2.5 s$^{-1}$ (k$_{\text{f}}$) and 0.007 s$^{-1}$ (k$_{\text{a}}$), respectively. Considering the fact that the rate constant obtained from the diode array experiment with the enzyme-product complex describes the entire reduction reaction as a monophasic process, the value of 2.5 s$^{-1}$ for k$_{\text{f}}$ is in rather good agreement with the single wavelength data that described the process by terphasic reaction kinetics (Table IV).

**Oxidation of Reduced 2-Hydroxybiphenyl 3-Monoxygenase**—Upon mixing of the reduced enzyme with air-saturated buffer, the flavin absorbance changed from the reduced spectrum to the oxidized spectrum in a fast biphasic reaction. Interestingly, at 30 and at 7 °C different kinetics were observed at different wavelengths (Fig. 5A). The highest rate for the increase of the initial absorbance was observed at 390 nm, which suggests that a spectral species with absorption characteristics around 390 nm was formed during the oxidation of the enzyme. However, no evidence for the stabilization of an intermediate was obtained when the reaction was analyzed with diode array detection (Fig. 5B). Moreover, it was impossible to generate the fully reduced spectrum of the flavin due to the dead time of the instrument (6 ms). From these results it is concluded that formation and decay of the putative flavin C(4a)-hydroperoxide were too fast to be detected.

**Oxidation of the Reduced Enzyme-Substrate Complex**—Next, reduced enzyme-substrate complex was mixed with air-saturated buffer in the stopped-flow spectrophotometer at 7°C. As can be seen from Fig. 6, most traces recorded at different wavelengths showed shapes that are indicative for the transient formation of at least one intermediate. Similar experiments performed with diode array detection were aimed at verifying putative flavin-oxygen adducts (Fig. 7A). Deconvolution analysis of spectral data on the basis of a three-step con-

**Table III**

Kinetic evidence for the formation of a charge transfer complex during anaerobic reduction of the enzyme-substrate complex of 2-hydroxybiphenyl 3-monoxygenase

In separate experiments, the free enzyme and the enzyme-product complex were anaerobically mixed with 2 mM NADH in 50 mM HEPES at a pH of 7.5. The reduction was followed at 450 nm in the stopped-flow spectrophotometer. Analogous experiments were performed with 5 mM NAD$^+$ present in both syringes. Symbols: k$_{\text{f}}$ and k$_{\text{a}}$, apparent first-order rate constants for the formation of the charge-transfer complex (cf. Scheme 3). Two or three first-order rate constants are shown in cases where multiphasic reaction kinetics were observed (see text for explanations).

**Table IV**

Effect of NAD$^+$ on the anaerobic reduction of the free enzyme and the enzyme-product complex by NADH

| Ligand-enzyme complex | T (°C) | NAD$^+$ absent, k$_{\text{f}}$ (s$^{-1}$) | NAD$^+$ present, k$_{\text{f}}$ (s$^{-1}$) |
|-----------------------|--------|-------------------------------------|-------------------------------------|
| Free enzyme           | 30     | 1.4*                               | 0.97                                |
|                       | 7      | 0.36                                | 0.21                                |
| 2,3-Dihydroxybiphenyl | 7      | 0.04                                | 0.04                                |
| | 1.4                          | 0.89                          | 0.02                                |

*cf. inset of Fig. 2A.
The consecutive reaction sequence revealed the presence of two spectral intermediates (Fig. 7B). By comparing the spectra of 2-hydroxybiphenyl 3-monooxygenase with the spectra of known oxygenated flavin intermediates (for instance those of p-hydroxybenzoate hydroxylase (13) and phenol hydroxylase (19)), the two intermediates observed during the oxidation of the enzyme-substrate complex of 2-hydroxybiphenyl 3-monooxygenase were assigned to the flavin C(4a)-hydroperoxide (intermediate I) and the flavin C(4a)-hydroxide (intermediate III), respectively. Therefore, the sequential formation of the two intermediates is consistent with the Scheme 4 as follows.

\[
\begin{align*}
&\text{E} \sim \text{FADH}_2 \text{S} + \text{O}_2 \rightarrow \text{E} \sim \text{FADHOOH} \text{S} \rightarrow \text{E} \sim \text{FADHOH} \text{P} \rightarrow \text{E} \sim \text{FAD} + \text{P} + \text{H}_2\text{O} \\
&k_1 \quad k_2 \quad k_3 \quad k_4
\end{align*}
\]

**Scheme 4. Oxidative half-reaction for 2-hydroxybiphenyl-3-monooxygenase complexed with 2-hydroxybiphenyl.**

The first phase of the reaction, which was identified by deconvolution analysis of the spectral data, was very short (completed within 30 ms) and linearly dependent on the concentration of oxygen. The reaction occurred at a second-order rate constant of \( 7.1 \times 10^5 \text{ M}^{-1} \text{ s}^{-1} \) (Fig. 7C, Table V). Therefore, this step represented the reaction of reduced flavin with oxygen yielding the flavin C(4a)-hydroperoxide (intrinsic molar absorption coefficient \( \varepsilon = 7.2 \text{ mM}^{-1} \text{ cm}^{-1} \)). The next phase, which was completed after 200 ms, displayed a first-order rate of \( 10.3 \text{ s}^{-1} \) and was represented by the formation of the flavin C(4a)-hydroxide (intrinsic molar absorption coefficient \( \varepsilon = 7.8 \text{ mM}^{-1} \text{ cm}^{-1} \)). The final phase, which took about 2.5 s to complete, showed a first-order rate of \( 2.7 \text{ s}^{-1} \) and was attributed to the decay of the flavin C(4a)-hydroxide intermediate. In accordance with the consecutive model depicted in Scheme 4, the latter two steps in the oxidative half-reaction were independent of the oxygen concentration.

Lowering the pH of the reaction to a value of 6.6 neither influenced the rate constants significantly nor improved the resolution of the processes involved in the oxidative half-reaction (Table V). Moreover, the spectra of the flavin-oxygen intermediates were qualitatively the same as at a pH of 7.5.

With 2-sec-butylphenol as the ligand, similar spectra were recorded as with 2-hydroxybiphenyl, and the rate constants of the individual reaction steps were of the same magnitude.
Hence, the existence of two intermediates during the oxidation of reduced 2-hydroxybiphenyl 3-monooxygenase was confirmed. From the kinetic data listed in Table V, it is evident that the decay of the flavin C(4a)-hydroxide was the slowest step in the oxidative half-reaction.

**Oxidation of the Reduced Enzyme-Product Complex**—No spectral intermediates were observed during the oxidation of the reduced enzyme-product complex. The absorbance change from the reduced to the oxidized flavin followed biphasic kinetics, and reoxidation was even faster than measured with the free enzyme (Table V). In the single wavelength mode the highest rate for initial absorbance increase was observed at 390 nm, which points to the transient formation of the flavin C(4a)-hydroperoxide. In the presence of 2,3-dihydroxybiphenyl this intermediate, which is necessarily formed upon reaction of reduced flavin with oxygen (11), decayed faster than in the free enzyme. Moreover, in the presence of 2,3-dihydroxybiphenyl no indication for the formation of the flavin C(4a)-hydroxide was found, which is consistent with the fact that 2,3-dihydroxybiphenyl is not further hydroxylated by 2-hydroxybiphenyl 3-monooxygenase (2). Although 2,3-dihydroxybiphenyl stimulates flavin reduction (cf. Table II), its binding to the reduced enzyme obviously hampers the stabilization of the flavin C(4a)-hydroperoxide.

**Oxidation of the Reduced Enzyme-Substrate Complex in the Presence of Monovalent Anions**—Because monovalent anions are known to affect the kinetics of flavoprotein monoxygenases (7), in particular of phenol hydroxylase (8, 19), it was of interest to study the oxidative half-reaction of 2-hydroxybiphenyl 3-monooxygenase in the presence of azide and chloride ions. Sodium azide did not change the optical flavin spectrum of the oxidized enzyme-substrate complex. When dithionite-reduced 2-hydroxybiphenyl 3-monooxygenase (21.8 \mu M) in complex with 0.2 mM 2-hydroxybiphenyl was reacted with air-saturated buffer in the presence of 0.2 M sodium azide at 7 °C, pH 7.5, the formation of the flavin C(4a)-hydroperoxide was enhanced. This is concluded from the fact that in diode array detection, the spectrum of the reduced enzyme-substrate complex was absent from the recordings even when the reaction was followed at a time scale of 0.96 s. The first spectrum taken at 5.6 ms after mixing resembled a composite of two flavin intermediates that were also observed in analogous experiments with the enzyme-substrate in the absence of azide (Fig. 8; cf. Fig. 7A). According to the reaction in Scheme 4, we calculated a series of composite spectra on the basis of the intermediate spectra shown in Fig. 7B to elucidate the composition of the spectra observed in the presence of azide (inset of Fig. 8). The first spectrum taken at 5.6 ms could be perfectly described as a composite of the spectrum of the flavin C(4a)-hydroperoxide, the spectrum of the flavin C(4a)-hydroxide, and the spectrum of the oxidized flavin. This suggests that, in the presence of azide, the reaction of the reduced enzyme-substrate complex with oxygen bifurcated to directly form oxidized enzyme from the flavin C(4a)-hydroperoxide, as well as the two flavin oxygen adducts. Due to the stabilization of the flavin C(4a)-hydroxide, the formation of the oxidized enzyme appeared to be a rather slow process (0.07 s⁻¹), taking about 1 min to complete. Despite this stabilization, there was no indication for the formation of the so-called intermediate II, which is characterized by a high extinction coefficient at 385 nm (34).

In the presence of 0.1 M sodium chloride, the formation of two distinct flavin intermediates was determined by deconvolution analysis of spectral data. The flavin C(4a)-hydroperoxide was formed with an apparent second order rate of 5.3 \times 10⁻³ M⁻¹ s⁻¹ and decayed with a rate of 7.4 s⁻¹ to form the flavin C(4a)-hydroxide. The latter slowly decayed to oxidized enzyme with a rate of 0.34 s⁻¹. The intermediate spectra were qualitatively similar to those observed in the absence of monovalent anions. Moreover, in contrast to p-hydroxybenzoate hydroxylase (7) and phenol hydroxylase (8), the absorption spectrum of the oxidized enzyme-substrate complex of 2-hydroxybiphenyl 3-monooxygenase was not changed in the presence of 0.1 M sodium chloride.

**DISCUSSION**

Monoxygenases acting on phenolic compounds constitute a prominent subgroup of the family of flavoprotein aromatic hydroxylases (10). Mechanistic studies on these environmentally important enzymes thus far were restricted to phenol hydroxylase from the basidiomycetous yeast *Trichosporon cutaneum* (8, 19). 2-Hydroxybiphenyl 3-monooxygenase from the soil bacterium *P. azelaica* HBP1 is the only flavoprotein aromatic hydroxylase described to date that acts on a bicyclic compound as the natural substrate (2). This and the efficient expression of the *hbpA* gene in *E. coli* prompted us to study the catalytic mechanism of this enzyme by rapid reaction techniques. The results from studies on the reductive and oxidative half-reactions of 2-hydroxybiphenyl 3-monooxygenase with different aromatic substrates and non-substrate effectors clearly established that the enzyme shares many mechanistic features with other flavoprotein aromatic hydroxylases. However, several unique kinetic properties were uncovered, which will be discussed in more detail below.

Anaerobic reduction of uncomplexed 2-hydroxybiphenyl 3-monooxygenase by NADH was a relatively slow process. However, the NADH-dependent reduction of the substrate-free enzyme occurred at a significant rate, in agreement with the NADH-oxidase activity previously observed from steady-state experiments (2). Anaerobic reduction of 2-hydroxybiphenyl 3-monooxygenase by NADH was strongly stimulated in the presence of aromatic ligands, acting as effectors. The rate of flavin reduction in the presence of the physiological substrate 2-hydroxybiphenyl was more than 1 order of magnitude higher than the overall turnover rate of the enzyme (2), which revealed that reduction of the enzyme-substrate complex by NADH is not limiting the rate of overall catalysis. Similar results including a significant NADPH oxidase activity were reported for 4-hydroxyphenylacetate 3-hydroxylase (15) and phenol hydroxylase (8, 18). In contrast, free p-hydroxybenzoate hydroxylase shows hardly any NADPH oxidase activity, and...
binding of the aromatic substrate to this enzyme results in a rate enhancement of flavin reduction of more than 4 orders of magnitude (12, 23).

Anaerobic reduction of substrate-free 2-hydroxybiphenyl 3-monoxygenase, the enzyme-2-sec-butylphenol complex, and the enzyme-2,3-dihydroxybiphenyl complex by NADH showed biphasic kinetics. Based on the equal contribution of the enzyme population to both phases, we have interpreted the biphasic kinetics as the existence of distinct enzyme forms. Moreover, as no indications were obtained for subunit dissociation (2), the biphasic kinetics most probably reflect different conformers of the tetrameric oxidized enzyme. If this interpretation is correct, the monophasic kinetics observed in the presence of 2-hydroxybiphenyl might indicate that the conformational state of the enzyme is dependent on the type of substrate bound and that binding of the physiological substrate induces the conformation which promotes efficient reduction. In this respect it is interesting to note that different FAD conformations have been detected in p-hydroxybenzoate hydroxylase (35–37) and that the mobility of the isoalloxazine ring of the flavin in this enzyme is presumed to play a crucial role in coenzyme recognition (9, 37–39). Moreover, recent studies from x-ray crystallography have pointed to the existence of open and closed forms of phenol hydroxylase (40). From this it was suggested that NADPH binding requires the open conformation with the FAD moved out of the hydroxylation site (40).

As suggested by one of the referees, kinetic experiments on the binding of substrates to the oxidized enzyme might give more insight in the possibility that 2-hydroxybiphenyl 3-monoxygenase exists in two conformational states that appear to be interconvertible. Preliminary results indeed indicate that at 30 °C, only part of the enzyme molecules rapidly interact with 2-hydroxybiphenyl and 2-sec-butylphenol. Moreover, the apparent rate constants for the formation of the enzyme-substrate complexes approach a limiting value \( k_{\text{obs}} \approx 100 \text{ s}^{-1} \) at high substrate concentrations, indicative of a mechanism involving a two-step equilibrium process (41). Certainly, further work on the structural and biological basis of this phenomena should be undertaken.

Anaerobic reduction of 2-hydroxybiphenyl 3-monoxygenase enzyme-ligand complexes by NADH involved the stabilization of a transient flavin charge-transfer species. In the presence of excess NAD⁺, the charge transfer absorption persisted for minutes, providing evidence that this long wavelength absorption reflects the ternary complex between reduced enzyme, substrate (or product), and NAD⁺. To our best knowledge, this high degree of stabilization is exceptional among flavoprotein aromatic hydroxylases.

During the reaction of the substrate-free reduced enzyme with oxygen, no stabilization of the putative flavin C(4a)-hydroperoxide was observed. With the notable exception of 4-hydroxyphenylacetate 3-hydroxylase (15), this is a common substrate complex, respectively. The data were fitted to a model with three consecutive reactions \( A \rightarrow B \rightarrow C \rightarrow D \). The inset shows a simulation of the concentrations as a function of time of the four components during the oxidation of reduced enzyme. C. The effect of the oxygen concentration on the rate constant for the formation of the first spectral intermediate was followed by diode array detection at 7 °C. A solution of 18.9 \( \mu \)M reduced enzyme in complex with 0.2 \( \mu \)M 2-hydroxybiphenyl was reacted with 50 \( \mu \)M HEPES at a pH of 7.5 containing different concentrations of oxygen. Solutions containing 10, 20, and 30% oxygen were produced by mixing appropriate volumes of 100% oxygen-saturated buffer and anoxic buffer, respectively. All buffer mixtures contained 0.2 \( \mu \)M 2-hydroxybiphenyl. The apparent first-order rate constants were plotted against the oxygen concentration. From the slope a second-order rate constant of 7.1 \( \times \) 10⁸ M⁻¹ s⁻¹ was calculated for the oxidation of the reduced enzyme-substrate complex.
Catalytic Mechanism of 2-Hydroxybiphenyl 3-Monoxygenase

TABLE V
Kinetic parameters for the oxidative half-reaction of 2-hydroxybiphenyl 3-monoxygenase

| Enzyme (complex)                  | T °C | pH  | \( k'_{\text{obs}} \) \( s^{-1} \) | \( k_4 \) \( s^{-1} \) | \( k_5 \) \( s^{-1} \) | \( k_6 \) \( s^{-1} \) |
|----------------------------------|-----|-----|-------------------------------|----------------|----------------|----------------|
| Free enzyme                      | 30  | 7.5 | \( 4.4 \times 10^5 \)          | 7.1 \( 10^5 \) | 10.3            | 2.7            |
| 2-Hydroxybiphenyl complex        | 7   | 7.5 | \( 1.6 \times 10^5 \)          | 6.4 \( 10^5 \) | 9.3             | 2.0            |
| 2-sec-Butylphenol complex        | 30  | 7.5 | \( 2.2 \times 10^5 \)          | 4.4 \( 10^5 \) | 9.9             | 2.5            |
| 2,3-Dihydroxybiphenyl complex    | 7   | 7.5 | \( 7.8 \times 10^4 \)          | 4.4 \( 10^5 \) | 14.4            | 14.4           |

\[ \text{Apparent rates for the overall reaction (} \left( k'_{\text{obs}} \right) \text{ in Scheme 4) were obtained from single wavelength reaction traces recorded at 450 nm.} \]

The lack of stabilization of the flavin C(4a)-hydroperoxide is not due to impaired binding of the aromatic product. A possible explanation is that binding of 2,3-dihydroxybiphenyl introduces steric constraints near the C(4a) atom of the isoalloxazine ring of the FAD, thereby promoting the rapid decomposition of the flavin C(4a)-hydroperoxide. A similar conclusion was drawn for the oxygen reaction of the reduced enzyme-product complex of p-hydroxybenzoate hydroxylase (32). Two consecutive spectral intermediates were observed when the oxidative half-reaction of 2-hydroxybiphenyl 3-monoxygenase was studied in the presence of 2-hydroxybiphenyl or the substrate analog 2-sec-butylphenol. Based on the studies of Massey and colleagues (11–15, 17–21, 46) with several other flavoprotein monoxygenases, these spectral intermediates were assigned to the flavin C(4a)-hydroperoxide (intermediate I) and flavin C(4a)-hydroxide (intermediate III), respectively. The conversion of intermediate I to intermediate III reflects the transfer of the terminal oxygen atom of the flavin C(4a)-hydroperoxide to the aromatic substrate with concomitant product formation (24).

With 2-hydroxybiphenyl 3-monoxygenase (at 7 °C), substrate hydroxylation was far more rapid than the decomposition of the flavin C(4a)-hydroxide and clearly not limiting the turnover rate. The absorption spectrum of intermediate III as obtained from the deconvolution analysis showed a shoulder near 480 nm (Fig. 7A), indicative for the presence of oxidized flavin (11, 17). As no such shoulder was found in the calculated spectrum of intermediate I (Fig. 7A), the most obvious interpretation is that in the presence of 2-hydroxybiphenyl (or 2-sec-butylphenol) part of the flavin C(4a)-hydroperoxide (about 20%) bifurcates to oxidized enzyme and hydrogen peroxide (Scheme 4). This unproductive competing side reaction is in good agreement with results from steady-state oxygen uptake experiments, which showed that all 2-hydroxybiphenyl 3-monoxygenase substrates partially uncouple oxygen activation from substrate hydroxylation (2).

Under the conditions studied, the presence of monovalent anions did not improve the kinetic resolution of reaction intermediates. However, in analogy with p-hydroxybenzoate hydroxylase (11, 13, 27) and phenol hydroxylase (19, 34), the decay of the C(4a)-hydroxyflavin to oxidized enzyme was considerably retarded. The decay of intermediate III was not affected by binding of excess substrate as it was observed for phenol hydroxylase (34). A likely explanation is that elimination of H₂O from the flavin C(4a)-hydroxide is preceded by product release. From studies on p-hydroxybenzoate hydroxylase and phenol hydroxylase with certain substrates, it has
been proposed that the formation of the non-aromatic hydroxy-
cyclohexadienone product and the flavin C(4a)-hydroxide (in-
termediate II) actually represents the hydroxylation step (24).
In the present work, no indication was obtained for the for-
mation of intermediate II in the catalytic mechanism of 2-hydroxy-
biphenyl 3-monooxygenase. It cannot be excluded that this is
simply due to the instability of the bicyclic hydroxycyclohexa-
diene products, which would result in rapid rearomatization.
Our studies do also not allow us to make a statement about the
protonation state of the flavin C(4a)-hydroperoxide and re-
duced enzyme-bound substrate (47). However, all the evidence
presently available suggests that 2-hydroxybiphenyl 3-mo-
noxygenase is a typical flavoprotein aromatic hydroxylase and
that hydroxylation of the bicyclic substrate involves the elec-
trostatic attack of the distal oxygen of the flavin C(4a)-hy-
droperoxide onto the π electrons of the activated aromatic ring.

In conclusion, this study has clearly demonstrated that the
aromatic substrate in 2-hydroxybiphenyl 3-monooxygenase has
strict control acting as an effector in both the reductive and
oxidative half-reaction. The work described here provides a
sound basis for future studies aimed at a better understanding
of the molecular principles of this control.

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