Mechanism of the 6-Hydroxy-3-succinoyl-pyridine 3-Monoxygenase Flavoprotein from Pseudomonas putida S16

Hao Yu, Robert P. Hausinger, Hong-Zhi Tang, and Ping Xu

From the 1 State Key Laboratory of Microbial Metabolism, and School of Life Sciences and Biotechnology, Shanghai Jiao Tong University, Shanghai 200240, People’s Republic of China and the 2 Department of Microbiology and Molecular Genetics, Michigan State University, East Lansing, Michigan 48824

Background: Mechanistic information is limited on pyridine β-hydroxylation monoxygenases.

Results: The catalytic mechanism of HspB was determined.

Conclusion: An observable C(4a)-hydroperoxyflavin intermediate reacts with 6-hydroxy-3-succinoyl-pyridine to form 2,5-dihydroxypyridine and succinate during the catalysis.

Significance: This study expands our understanding of pyridine hydroxylases and their pyridine metabolisms.

6-Hydroxy-3-succinoyl-pyridine (HSP), a flavoprotein essential to the pyridine pathway of nicotine degradation, catalyzes pyridine-ring β-hydroxylation, resulting in carbon-carbon cleavage and production of 2,5-dihydroxypyridine. Here, we generated His6-tagged HspB in Escherichia coli, characterized the properties of the recombinant enzyme, and investigated its mechanism of catalysis. In contrast to conclusions reported previously, the second product of the HspB reaction was shown to be succinate, with isotope labeling experiments providing direct evidence that the newly introduced oxygen atom of succinate is derived from H2O. Phylogenetic analysis reveals that HspB is the most closely related to two p-nitrophenol 4-monoxygenases, and the experimental results exhibit that p-nitrophenol is a substrate of HspB. The reduction of HspB (with maxima at 375 and 460 nm, and a shoulder at 485 nm) by NADH was followed by stopped-flow spectroscopy, and the rate constant for reduction was shown to be stimulated by HSP. Reduced HspB reacts with oxygen to form a C(4a)-hydroperoxyflavin intermediate with an absorbance maximum at ~400 nm within the first few milliseconds before converting to the oxidized flavoenzyme species. The formed C(4a)-hydroperoxyflavin intermediate reacts with HSP to form an intermediate that hydrolyzes to the products 2,5-dihydroxypyridine and succinate. The investigation on the catalytic mechanism of a flavoprotein pyridine-ring β-position hydroxylase provides useful information for the biosynthesis of pyridine derivatives.

6-Hydroxy-3-succinoyl-pyridine (HSP) 3-monoxygenase (HspB), encoded by the first gene in the nic2 cluster involved in the pyrrolidine pathway of nicotine degradation in Pseudomonas putida strain S16, is a flavin-dependent monoxygenase (1 – 4) that contains an FAD cofactor and depends on NADH as a co-substrate. This pyridine-ring β-hydroxylation monoxygenase (Fig. 1A) is responsible for converting HSP to 2,5-dihydroxypyridine (2,5-DHP) (1), a precursor for the important drug 5-aminolevulinic acid that is used for cancer therapy (5).

Pyridine derivatives are extensively used in functional materials and found in natural products that possess important biological activities. Organic chemical synthesis of pyridine compounds is commonly accompanied by the formation of by-products (6 – 8), resulting in prohibitively high costs for the pure chemicals; thus, biocatalysis is an attractive alternative synthetic technology, allowing in some cases reactions that are not easily conducted by classical organic chemistry (9).

Information on biocatalytic enzymes involved in the metabolism of pyridine derivatives is sparse (10) despite their ubiquity in the environment (11).

Pyridine-ring hydroxylations can be subdivided according to whether they catalyze α-, β-, or γ-hydroxylation of the pyridine ring (Fig. 1A). In contrast to benzene, the electron density is unevenly distributed over the pyridine ring, which thus influences the positional reactivity and the interaction with enzymes. Most of the reported pyridine ring β-hydroxylation monoxygenases are multicomponent molybdenum-containing proteins (12 – 14). In addition to HspB (EC 1.14.13.163), two other flavin-dependent pyridine-ring β-hydroxylation monoxygenases have been isolated and studied: 6-hydroxynicotinate 3-monoxygenase (6HN3M, EC 1.14.13.114) (12, 15) and 2,6-dihydroxypridine 3-monoxygenase (DHPH, EC 1.14.13.10) (16) (Fig. 1A). These three monoxygenases have low sequence identities to each other (Fig. 2), and either they are unable to transform the corresponding homocyclic analogs or they exhibit low activity. Significantly, mechanistic information is limited on pyridine β-hydroxylation monoxygenases; e.g. no catalytic mechanism has been reported for any of these enzymes.
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Flavin-dependent monooxygenases constitute a large family of flavoenzymes, and more than 130 members have been described since 1957 (17). These enzymes have been classified into eight groups, labeled A through H. A phylogenetic analysis of selected flavin-dependent monooxygenases (Fig. 2) reveals that HspB is associated with the group A enzymes, even though it contains two Rossmann-folds (i.e. nucleotide binding sites with the characteristic “GXGXXG(X)_{16–17}(E/D)” motif), which is a property of group B flavin-dependent monooxygenases. HspB exhibits the highest sequence identity with the flavoenzymes that use non-pyridine-containing substrates: PnpA from Pseudomonas sp. WBC-3 (18), PnpA2 from P. putida DLL-E4 (19), and 4-hydroxyphenylacetate 1-monooxygenase (4HPA1M) from Ralstonia solanacearum (20). These enzymes, along with p-hydroxybenzoate 1-monooxygenase (PHB1H), catalyze ring hydroxylations para to an existing hydroxyl group (Fig. 1B), similar to the pyridine ring β-hydroxylases noted above (Fig. 1A).

Flavin-dependent monooxygenases have catalytic reactions that involve two steps: a reductive half-reaction and an oxidative half-reaction (21–25). The enzyme-FAD complexes are first reduced by NAD(P)H, and the reduced enzymes then react with oxygen to form a $C_{(4a)}^\cdot$ (hydro)peroxyflavin species. These oxidants have been proposed to react with the substrates, resulting in the formation of products and the $C_{(4a)}^\cdot$-hydroxyflavin that dehydrates to the oxidized flavin. Flavin-dependent monooxygenases have been studied for over 40 years, and many of these enzymes are well understood in terms of structures, kinetics, and mechanisms (21, 26, 27). Nevertheless, additional enzymes with new biochemical properties are likely to be uncovered, particularly those involved in the metabolism of pyridine derivatives.

In this study, we present the characterization of heterologously expressed His-tagged HspB (His-HspB) from Escherichia coli cells. The second product of HspB reaction, previously suggested to be succinate semialdehyde (SSA) (1), is shown here to be succinic acid with the added oxygen atom derived from solvent rather than $O_2$. Transient kinetics of the reductive and oxidative half-reactions of His-HspB in the presence and absence of HSP (as well as with the product 2,5-DHP and the alternate substrate p-nitrophenol) were investigated to reveal mechanistic insights about the unique reaction catalyzed by this flavoprotein. A scheme for the catalytic mechanism is proposed. This study establishes key features of the catalytic mechanism of HspB that will likely apply to related enzymes, thus offering a new catalytic potential for the metabolism of pyridine derivatives.

**EXPERIMENTAL PROCEDURES**

**Materials**—HSP was produced as previously described (28). FAD, NADH, and NAD$^+$ were purchased from Sigma. 2,5-DHP was purchased from SynChem OHG (Kassel Corp., Kassel, Germany). $H_2^{18}O$ was obtained from the Shanghai Research Institute of Chemical Industry (Shanghai, China). All other regents and solvents used in this study were of analytical grade and commercially available. *P. putida* S16 can be obtained from the German Culture Collection (DSMZ, Braunschweig, Germany) under the accession number DSM 28022.

**Overexpression and Purification of Heterologously Expressed His-HspB**—The DNA fragment containing *hspB* was amplified by Pfu DNA polymerase (New England Biolabs, Ipswich, MA) using DNA primers *hspB*-H-F (ATACCATGGTGAGCATGGAGCA-TGAAAAACGCGGTAAT) and *hspB*-H-R (GTGTCCGAGAAAGTTTCCATAGTCTCGGAA). PCR products, treated with NcoI and XhoI, were ligated into the expression vector pET28a (Invitrogen), which was treated with the same NcoI and XhoI enzymes for heterologous expression. Recombinant *E. coli* strains were grown at 37 °C in Luria-Broth medium containing 50 μg of kanamycin/ml. The culture was induced by adding 0.2 mM isopropyl β-D-thiogalactopyranoside until reaching an $A_{600\text{ nm}}$ between 0.6 and 0.8, and then the culture was grown at 16 ºC for 10 h. *E. coli* cells were harvested by centrifugation and re-suspended in 20 mls Tris-HCl (pH 8.0) buffer. Cells were disrupted by ultrasonication and His-HspB was purified on a His-Trap column using a liquid chromatography system (AKTA, GE Healthcare) following standard procedures. The eluted sample was loaded onto a
Superdex 200 column that had been pre-equilibrated with 20 mM Tris-HCl (pH 8.0) buffer. The collected His-HspB was preserved at −70 ºC for further study.

Enzyme Assays—To test the in vitro activity of His-HspB, HSP and NADH were mixed with the recombinant enzyme in air-saturated 20 mM Tris-HCl buffer (pH 8.0) at 25 ºC. The decrease in absorbance at 340 nm, which was monitored by using a UV-2550 spectrophotometer (Shimadzu, Kyoto, Japan) or a 96-well plate reader (Victor® X3, 2030 Multilabel Reader, PerkinElmer Life Sciences), was used to calculate the enzyme activity of HspB. The activity of His-HspB with 3-succinoylpyridine (SP) was determined under the same conditions. In some experiments His-HspB powder was used, obtained by using vacuum freeze-drying methods.

Anerobic reduction of protein-bound FAD was performed in an anaerobic work station AW200SG (Electrotek, Sheffield, UK). All compounds were dissolved in oxygen-free buffer (20 mM Tris-HCl, pH 8.0). Spectra were recorded using a UV-2550 spectrophotometer.

To estimate the uncoupling reaction of His-HspB with (or without) substrates, hydrogen peroxide production was measured. Specifically, 100 µl of enzyme reaction mixture (1 µM enzyme, 250 µM HSP, 200 µM NADH, in Tris-HCl buffer (pH 8.0) with continuous aeration) was terminated after 5 min and combined with 700 µl of detection solution (5 mM 4-aminoanitopyrene, 10 mM vanillic acid, and 40 units ml−1 horseradish peroxidase in 0.2M NaCl and 0.2M MOPS (pH 7.5)). The combined mixture was incubated at 37 ºC for 5 min. The absorbance was recorded at 498 nm to detect the product resulting from the generation of H2O2.

Product Analysis and18O Isotope Experiments—The products of the His-HspB reaction, which was terminated by addition of 9 volumes of methanol, were confirmed by HPLC and reanalyzed by liquid chromatography-mass spectrometry (LC-
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With electro spray ionization (ESI) (1). Negative and positive ion ESI mass spectra were both obtained to detect HSP, succinate, and 2,5-DHP.

To confirm the source of the oxygen atom incorporated into succinate, H218O was used as a solvent for the His-HspB reaction. An assay mixture containing His-HspB dried powder and substrates was dissolved in H218O and incubated at room temperature for 10 min. After termination of the reaction by adding 9 volumes of methanol, samples were analyzed using LC-ESI-MS.

**Rapid Reaction Experiments**—Reactions were carried out in 50 mM Tris-HCl (pH 7.5) buffer at 4 °C, unless otherwise specified. Rapid kinetics measurements were performed with a SFM-4000 stopped-flow apparatus (Bio-logic, France) installed in an anaerobic glove box. Temperature was controlled by using a temperature-controlled bath JULABO model F12 (Julabo, Seelbach, Germany). Spectral scans were recorded with a TIDIS S 300K diode array detector (J&M Analytik AG, Germany). The optical path length was 1 cm. Oxygen in the solutions was removed by flushing the flow system with an anaerobic buffer solution containing 40 units ml−1 of glucose oxidase, 400 units ml−1 of catalase, and 100 mM glucose in 50 mM Tris-HCl (pH 7.5) buffer. Apparent rate constants (kobs) were calculated by fitting the kinetic traces to a sum of two exponentials using the software program Origin 8.0. Changes in flavin absorption were monitored at 2.2-nm intervals from 200 to 700 nm.

To study the kinetics of reduction of the enzyme by NADH, a solution of oxidized His-HspB in the presence or absence of HSP (or 2,5-DHP or p-nitrophenol (PNP)) with 100 mM glucose, 40 units ml−1 of glucose oxidase, and 400 units ml−1 of catalase was loaded into the stopped-flow instrument. The enzyme complex was mixed (in a 1:1 ratio) with varied concentrations of NADH prepared in anaerobic 50 mM Tris-HCl (pH 7.5) buffer with 100 mM glucose, 40 units ml−1 of glucose oxidase, and 400 units ml−1 of catalase. All concentrations of substrates, product, and NADH used for rapid kinetic studies were at least 7-fold higher than the enzyme concentration to maintain pseudo-first order conditions. All absorbance traces were monitored from 200 to 700 nm.

To study the oxidative half-reaction, His-HspB with or without HSP (or 2,5-DHP or PNP) was anaerobically reduced by 200 μM NADH or 200 μM Na2S2O4 and loaded into the stopped-flow equipment. The reduced enzyme complex was mixed with various concentrations of dioxygen (from 0.3 to 1.5 mM). The dioxygen solutions were prepared by sparging a solution of 50 mM Tris-HCl (pH 7.5) buffer with air or oxygen at 16 °C. All absorbance traces were monitored from 200 to 700 nm.

**Sequence Analysis of HspB**—Sequences of HspB and selected other flavin-dependent monoxygenases were aligned using CLUSTAL W software. A phylogenetic tree was constructed using the MEGA 5.0 program.

**Analytical Methods**—Analytical HPLC in this study was performed on an Agilent 2100 HPLC instrument (Agilent, Santa Clara, CA) equipped with an Eclipse XDB-C18 column (5 μm, 4.6 × 250 mm, Keystone Scientific, Bellefonte, PA), diode array detection, and a mobile phase consisting of 80% (v/v) 1 mM sulfuric acid and 20% (v/v) methanol at a flow rate of 0.5 ml min−1. Retention time and absorption spectra were compared with standard compounds for each run to identify the substrates or products. ESI-MS analysis was performed on an Agilent 6230 time-of-flight-MS equipped with ESI sources. Samples were diluted to the appropriate concentrations with methanol, filtered through a 0.22-μm membrane filter, and then loaded into the mass spectrometer in 40% (v/v) methanol (0.1% formic acid, v/v) and 60% (v/v) deionized water (18 megohm cm−1) (0.05% formic acid, v/v) at a flow rate of 0.2 ml min−1.

**RESULTS**

Characterization of Heterologously Expressed His-tagged HspB—We previously reported that HspB expression in the native strain of P. putida S16 is poor (~0.4% (w/w) of total protein) (1), precluding extensive mechanistic studies of the enzyme. Here, the highly expressed His-HspB version of the protein was purified to more than 95% homogeneity from E. coli cells in two steps and characterized (Fig. 3A). Apparent HSP Km and kcat values were 173 ± 6.78 μM and 7.74 ± 0.12 s−1 (Fig. 3B), using 250 μM NADH), respectively, for His-HspB. Apparent NADH Km and kcat values (Fig. 3C, using 250 μM NADH) were 29.4 ± 2.97 μM and 5.49 ± 0.34 s−1, respectively, for the tagged enzyme. Purified His-HspB was stored at ~80 °C as a frozen solution or dry powder with no change in activity for at least 3 months.

Product Analysis of the Oxidative Cleavage Reaction—The products of the reaction catalyzed by His-HspB were identified by comparison to the mass spectra (m/z) of standard compounds using ESI-MS. SSA, previously identified as a product of the enzymatic reaction (1), exhibits a [M-H]-deprotonated molecule m/z of 101.0246 (Fig. 4C); it is not oxidized to succinate by HspB in the presence or absence of HSP and NAD+ (Fig. 4, E and G). In contrast to the earlier interpretations, succinate ([M-H]-deprotonated molecule m/z 117.0194; Fig. 4B) was identified as the authentic second end product of HSP conversion to 2,5-DHP by His-HspB (Fig. 4F), with no SSA observed in the His-HspB-catalyzed reaction.

One oxygen atom is added to each of the two products (2,5-DHP and succinate) in the reaction of His-HspB. The source of the oxygen added to 2,5-DHP comes from the dioxygen added to 2,5-DHP, because no18O-
labeled 2,5-DHP was observed in the H$_2$O experiment (Fig. 4, J and K), and they showed that all of the succinate produced in H$_2$O contained one atom of $^{18}$O (Fig. 4, H and I). For comparison, samples of HSP dissolved in H$_2$O and H$_2$O both yielded spectra with $m/z$ 194.046 (Fig. 4, H and I), indicating a lack of substitution between the carbonyl of HSP and H$_2$O. These results demonstrate the oxygen added to succinate originates from H$_2$O rather than from exchange into HSP, indicating that H$_2$O is a substrate in the His-HspB-catalyzed reaction. The results of this labeling experiment lead us to conclude that the second oxygen atom of molecular oxygen, i.e. the one not added to product, is reduced to H$_2$O as in other flavin-dependent monooxygenase-catalyzed reactions (24–26). H$_2$O is therefore both a substrate and product of His-HspB.

**Substrate Specificity**—Five substrates known to be used by other flavin monooxygenases were examined as potential substrates of His-HspB: SP (a precursor of HSP, missing only the ring hydroxyl group; located in the nicotine degradation pathway used by strain S16), 2-hydroxypyridine, 2,6-dihydroxypyridine, and 6-hydroxynicotinate (substrates of the pyridine/hydroxylases DHPH and 6HN3M), and PNP (the substrate of PnpA). These compounds were tested as substrates of His-HspB by using the standard assay conditions for the HspB reaction. Of these compounds, His-HspB exhibited trace NADH consumption activity with PNP (Fig. 5A), but no activity was detected with other substances (data not shown). PnpA is known to convert PNP to quinone (Fig. 1B) (18, 29), which is then reduced to the hydroquinone by PnpB or non-enzymatically by using NADH (data not shown). Non-enzymatic conversion of PNP to hydroquinone by NADH was not observed (Fig. 5B). Furthermore, formation of hydroquinone was not detected.
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when using just His-HspB. In contrast, small amounts of NADH were transformed by the enzyme into hydroquinone in the additional presence of NADH according to HPLC determinations (Fig. 5B). The amounts of NADH and NADH consumed by His-HspB differ, indicating that some of the reducing potential of the NADH may be partitioned to other products. Indeed, H2O2 determinations (Fig. 5C) revealed that the His-HspB reaction with PNP generated more H2O2 than when the enzyme was incubated with only NADH. These results suggest that PNP stimulates peroxide release.

Spectral Properties of Oxidized HspB and Analysis of HSP Binding—His-HspB exhibits the spectrum expected of a flavoprotein with absorbance maxima at 375 and 460 nm along with a shoulder at ~485 nm (Fig. 6A). The His-HspB-catalyzed reaction was not accelerated by addition of free FAD to the assay mixture, indicating that the flavin cofactor is tightly bound to the protein (30). The addition of 0.1% (w/v) SDS to His-HspB for 10 min led to shifts in the spectrum that are representative of free FAD (Fig. 6A). Based on the spectrum, a molar absorption coefficient of 10,170 M⁻¹ cm⁻¹ was estimated for His-HspB at 460 nm and pH 8.0.

Static titration experiments were performed using 63 μM His-HspB and 0–1,600 μM HSP to study the interaction between the oxidized enzyme and substrate. Substrate addition led to increases in absorbance at 390 and 450 nm along with decreases at 499 nm (Fig. 6B). The calculated dissociation constant (K_D) of HSP was 480 ± 36.7 μM, indicating a weak interaction between oxidized His-HspB and HSP.

The kinetics of substrate binding to the oxidized protein was assessed by monitoring the absorbance changes at 441 nm (Fig. 7A) and data were fitted to a single exponential. The k_off derived from the kinetic traces shows a linear dependence on HSP concentration in the range of 0.2–2 mM, corresponding with a second-order rate constant for HSP binding to enzyme of $3.21 \pm 0.16 \times 10^{4}$ M⁻¹ s⁻¹ (Fig. 7B). The intercept of Fig. 7B provides an estimate for k_off of 6 s⁻¹, so the K_D can be estimated as k_off/k_on (~190 μM), which is in reasonable agreement with that measured by static methods.

Reduction of His-HspB by NADH—His-HspB was mixed with excess NADH or Na2S2O4 under anaerobic conditions. The resulting spectra both revealed complete reduction of the flavin (Fig. 6C). After all of the His-HspB-bound FAD (100 μM) was reduced by NADH or Na2S2O4, HSP (200 μM) was added anaerobically, and after 10 min the reactions were terminated. No transformation of HSP was detected by HPLC analysis, indicating that molecular oxygen is required for catalysis after the enzyme complex is first reduced by NADH.

To examine the kinetics of reduction of the flavin cofactor by NADH, equivalent volumes of oxidized His-HspB and NADH were mixed anaerobically by using a stopped-flow spectrophotometer. The absorbance of His-HspB decreased in a range from 300 to 500 nm, and the reduction reactions were monitored by the absorbance changes at 460 nm, a wavelength that provides the maximum difference between oxidized and reduced His-HspB (Fig. 8A, inset). The traces obtained for samples prepared under different conditions were best fitted to two-exponentials. The k_off for 48.4 μM His-HspB mixed with 20 mM NADH was 1.02 ± 0.08 s⁻¹ (Fig. 8A, line a). The inclu-
sion of product 2,5-DHP (2 mM) with enzyme prior to mixing with NADH had only a minor influence on the rate of reduction ($k_{\text{obs}}$ of 1.74 ± 0.06 s$^{-1}$); however, a pre-mixture of His-HspB with 2 mM concentrations of substrates HSP (line f) or PNP (line e) led to marked stimulations of the observed reduction rate constants by NADH ($k_{\text{obs}}$ of 152 ± 5 and 14.2 ± 0.6 s$^{-1}$, respectively) (Fig. 8A). Including HSP with both enzyme and NADH provided no further stimulation (line g, $k_{\text{obs}}$ of 146 ± 4 s$^{-1}$), and this substrate was less effective at stimulating reduction when pre-mixed only with NADH (line b, $k_{\text{obs}}$ of 29.8 ± 1.5 s$^{-1}$), whereas PNP was nearly as effective when mixed only with NADH (line c, $k_{\text{obs}}$ of 11.6 ± 0.9 s$^{-1}$). Overall, HSP is a better enhancer for HspB reduction compared with PNP, and the flavin reduction rate constant with saturating concentrations of NADH is stimulated by HSP by ~150-fold.

The $k_{\text{obs}}$ of the reduction of free enzyme with NADH and HSP (Fig. 8A, line b) was lower than that of the reduction of His-HspB that was premixed with HSP (lines f and g). In addition, the rate for binding of HSP (1 mM) to His-HspB (with $k_{\text{obs}}$ of ~36.9 s$^{-1}$) is faster than the rate of reduction of free His-HspB by NADH-HSP. These results suggested that formation of the His-HspB-HSP complex was required prior to binding of NADH to attain the maximal stimulation of reduction. HspB has evolved to prevent the consumption of NADH and oxygen unless HSP is bound to the enzyme.

The kinetics of His-HspB flavin reduction by various concentrations of NADH in the absence of substrate were examined by anaerobically monitoring the absorbance changes at 460 nm upon mixing (Fig. 8B). The $k_{\text{obs}}$ associated with the major phase
of reduction, accounting for ~95% of the total amplitude, exhibited a hyperbolic dependence on NADH concentration. The limiting value of $k_{\text{obs}}$ was $1.12 \pm 0.01$ s$^{-1}$ and the NADH concentration at half-saturation (i.e. the dissociation constant $K_{d}^{\text{NADH}}$) was $0.72 \pm 0.03$ mM. No evidence was observed for an absorption associated with a charge-transfer (CT) complex, typically observed in the 500–700 nm range.

To investigate the kinetics of flavin reduction for substrate-bound enzyme, solutions of oxidized His-HspB and HSP were anaerobically mixed with different concentrations of NADH while monitoring the absorbance changes at 460 nm (Fig. 9A). The predominantly monophasic reduction phases provided observed rate constants showing a hyperbolic dependence on NADH concentration. The limiting value of $k_{\text{obs}}$ and the calculated $K_{d}^{\text{NADH}}$ were $156 \pm 1$ s$^{-1}$ and $0.17 \pm 0.01$ mM, respectively. A CT complex was observed in the reductive half-reaction of His-HspB in the presence of HSP over the absorbance range of 500–700 nm (Fig. 9C). By contrast, no corresponding CT complex was detected without HSP or with product 2,5-DHP or the alternate substrate PNP.
The kinetics of CT formation (\(k_{\text{obs, formation}} = 382 \pm 90 \text{ s}^{-1}\)) and decay (\(k_{\text{obs, decay}} = 23.3 \pm 1.1 \text{ s}^{-1}\)) were assessed by monitoring the absorbance changes at 700 nm for the reduction reaction of His-HspB by NADH in the presence of HSP (Fig. 9B). The reduction of the flavin cofactor (characterized by a major decrease in absorbance at 460 nm) was accompanied by CT formation (resulting in a small increase at 700 nm) (Fig. 9, A and B). This was followed by decay of the CT complex after 20 ms.

Free \(H_2O_2\) Does Not Catalyze the His-HspB Reaction—In the absence of HSP, the reduced FAD cofactor of His-HspB is rapidly reoxidized by air, consistent with the enzyme acting as an NADH oxidase that generates \(H_2O_2\) with \(k_{\text{obs}}\) of (23.5 \pm 1.33) \times 10^{-4} \text{ s}^{-1} \) (when using 250 \(\mu\text{M}\) NADH and 17.6 \(\mu\text{M}\) HspB). To determine whether \(H_2O_2\) (as the \(E\)-FAD-NAD\(^+\)-\(H_2O_2\) complex) can serve as the oxygenating species for transformation of HSP, an anaerobic assay mixture containing His-HspB (15 \(\mu\text{M}\)), NAD\(^+\) (0.5 mM), \(H_2O_2\) (0.5 mM), and HSP (400 \(\mu\text{M}\)) was incubated at room temperature. After 10 min, the reaction was terminated under anaerobic conditions. No HSP transformation was detected by HPLC analysis (data not shown). His-HspB cannot catalyze the transformation of HSP with exogenous \(H_2O_2\), indicating that the flavin C(4a)- (hydro)peroxyflavin intermediate is probably the true oxygenating species. No \(H_2O_2\) was detected in the His-HspB-catalyzed reaction when using excess HSP under aerobic conditions (data not shown), indicating that HSP likely stabilizes the proposed C(4a)- (hydro)peroxyflavin intermediate and prevents its decomposition to free \(H_2O_2\).

Kinetics of Oxidation of His-HspB—In the oxidative half-reaction of flavin-dependent monoxygenases, the reduced enzyme reacts with oxygen to form a C(4a)- (hydro)peroxyflavin species that can persist from a few milliseconds to hours. The C(4a)-intermediates have been proposed to react with the substrates, resulting in the formation of products (26). To investigate the oxidative half-reaction of the HspB reaction, 48.4 \(\mu\text{M}\) His-HspB (reduced with 200 \(\mu\text{M}\) NADH or 200 \(\mu\text{M}\) Na\(_2\)S\(_2\)O\(_4\)) was mixed with equal volumes of oxygen-saturated buffers using a stopped-flow spectrophotometer under anaerobic conditions. No C(4a)- (hydro)peroxyflavin intermediate was detected in the absence of HSP (lines a and b of Fig. 10A). All wavelengths gave similar observed rate constants (data not shown). The results indicated that the putative C(4a)-intermediate formed too slowly or decays too rapidly to be detected for this condition. Rather, the reduced enzyme samples were directly transformed to the fully oxidized protein (\(k_{\text{obs}}\) of 22.2 \pm 0.3 and 23.1 \pm 1.0 \text{ s}^{-1}\), respectively).

The effects of various additives on the oxidative half-reaction were assessed. The presence of varied concentrations of NAD\(^+\) with Na\(_2\)S\(_2\)O\(_4\)-reduced His-HspB had no effect on the observed rate constant of flavin oxidation (\(k_{\text{obs}}\) of 23.1 \pm 1.0, 21.0 \pm 0.6, and 21.4 \pm 0.4 \text{ s}^{-1}\), for 0, 50 \(\mu\text{M}\), and 1 mM NAD\(^+\), respectively, Fig. 10B). These results imply that NAD\(^+\) binds very weakly to reduced HspB or likely leaves upon flavin reduction, as noted for other group A flavin-dependent monoxygenases (26, 31). Similarly, 2.5-DHP has no influence on the rate constant of His-HspB oxidation (\(k_{\text{obs}}\) of 23.3 \pm 1.3 \text{ s}^{-1}\), line e of Fig. 10A) and no spectral intermediates were observed during the oxidation reaction in the presence of this product. In contrast, provision of substrate HSP during the oxidation of NADH-reduced HspB yielded biphasic kinetics (line c, Fig. 10A), whereas the Na\(_2\)S\(_2\)O\(_4\)-reduced enzyme yielded similar kinetics whether HSP was present or not (compare lines b and d, Fig. 10A).

The kinetics of oxidation for NADH-reduced His-HspB in the presence of HSP was examined more extensively by analysis over the entire spectrum. Within the first milliseconds after mixing, an intermediate appeared with a peak at ~400 nm (Fig. 11, A and B). This species is consistent with the presence of a C(4a)- (hydro)peroxyflavin that in other flavin-dependent monoxygenases generally has an absorbance maximum between 360 and 410 nm. The C(4a)-intermediate was only observed in the presence of HSP, and was not detected with PNP or 2.5-DHP (data not shown) in the reaction. The results suggest that HSP stabilizes the C(4a)-intermediate. Although PNP stimulates the reduction of HspB, it does not appear to stabilize the C(4a)-intermediate; rather, as shown earlier (Fig. 5) PNP enhances the NADH oxidase activity of HspB.

The kinetics of His-HspB oxidation with oxygen-saturated buffer (1.5 mM) in the presence of HSP showed two phases.
Phase 1 (10–30 ms) was characterized by an increase in absorbance at 400 nm (Fig. 11C, bottom), representing the formation of the C(4a)-intermediate. Phase 2 (from 50 ms to the end of the reaction) was characterized by a secondary increase in absorbance at 460 nm (oxidized flavin) and a decrease in absorbance at 340 nm (consumption of excess NADH) (Fig. 11C, bottom). In the initial stage of phase 2 (50 ms-150 ms), a small decrease in absorbance at 400 nm was observed, accompanying the larger decrease in absorbance at 340 nm and the increase in absorbance at 460 nm. During this phase, the C(4a)-intermediate is proposed to be converted to oxidized His-HspB at a rate that is greater than the reduction of oxidized His-HspB by NADH. In the middle stage of phase 2 (150 – 600 ms), an equilibrium was established between the reduction and oxidative process. This situation led to no absorbance change at 400 and 460 nm; however, a major decrease in absorbance at 340 nm was observed. On the basis of the intensity of the absorbance at 460 nm during this stage, we conclude that these turnover conditions lead to ∼53% His-HspB being in the oxidized form. The oxidized flavin species were monitored at 400 nm (top) and 460 nm (bottom). The insets show that $k_{\text{obs}}$ values of C(4a)-intermediate formation and the oxidative reaction both exhibit hyperbolic dependence on oxygen concentration.

**FIGURE 11. Time-dependent spectral changes during oxidation of His-HspB in the presence of HSP.** All concentrations indicated here are final concentrations after mixing. A, absorbance spectra collected with mixing times ranging from 10 ms to 4 s for the oxidation of His-HspB (24.2 μM), reduced by 100 μM NADH, containing 1 mM HSP when mixed with an equal volume of oxygen-saturated buffer (50 mM Tris-HCl, pH 7.5). B, (top) formation of the C(4a)-intermediate showing an increase of absorbance at 400 nm. These difference spectra were calculated by subtracting the initial spectrum obtained at 1 ms. Bottom, consumption of NADH as shown by a decrease of absorbance at 340 nm. These difference spectra were calculated by subtracting the spectrum obtained at 100 ms. C, kinetics of the formation of the C(4a)-intermediate (400 nm), consumption of NADH (340 nm), and oxidation of the His-HspB flavin (460 nm). The oxidation reaction of His-HspB with oxygen-saturated buffer was performed in the absence (top) or presence (bottom) of HSP. D, a solution of His-HspB (24.2 μM) and HSP (1 mM), reduced by stoichiometric amounts of NADH, was mixed with an equal volume of buffer containing various concentration of oxygen. The oxidized flavin species were monitored at 400 nm (top) and 460 nm (bottom). The insets show that $k_{\text{obs}}$ values of C(4a)-intermediate formation and the oxidative reaction both exhibit hyperbolic dependence on oxygen concentration.

Mechanism of 6-Hydroxy-3-succinoyl-pyridine 3-Monoxygenase

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FIGURE 12. Proposed mechanism of the HspB-catalyzed reaction. In the reductive half-reaction, the FAD in HspB is reduced by NADH to form a reduced enzyme complex. In the first step of the oxidative half-reaction, the reduced flavin reacts with oxygen (red) to form a C(4a)-hydroperoxyflavin possibly with the intermediacy of a C(4a)-peroxyflavin that becomes protonated. A resonance structure of deprotonated HSP, with selected carbons labeled, having partial anionic character on C-3 of the pyridine ring reacts with the hydroperoxyflavin to yield intermediate 1. Hydration of the carbonyl group by a hydroxide shown in blue leads to intermediate 2, which is transformed through a retro-aldol mechanism to generate 2,5-DHP and succinate.

DISCUSSION

Flavin-dependent monooxygenases introduce a single oxygen atom into their substrates, so these enzymes are ideal for performing a critical step in the metabolism of pyridine derivatives by activating the heterocycle (26, 32, 33). In the case of HspB, the HSP-derived intermediate generated by oxygen addition decomposes by carbon-carbon bond cleavage that leads to release of 2,5-DHP and the four-carbon product. In this study, we identified succinate as this second product and showed by 18O-isotope labeling experiments that H2O serves as the source of the new oxygen atom incorporated into succinate. Here, we identified an alternative substrate of the enzyme that provides mechanistic insight and we investigated the reductive and oxidative half-reactions by transient kinetic analyses to elucidate the kinetic mechanism of HspB, thus providing an understanding of the mechanism of pyridine-ring β-hydroxylation monooxygenases.

One can envision three possible hydroxylation schemes for the conversion of HSP to 2,5-DHP and succinate. The first hypothesis invokes an initial hydrolysis step to produce succinate and 2-hydroxypyridine, with subsequent monooxygenation of the heterocycle. Such a sequence of reactions is analogous to the hydrolysis of 2,6-dihydroxypyseudooxonicotine followed by the flavoenzyme-catalyzed conversion of 2,6-dihydroxypyridine to 2,3,6-trihydroxypyridine in one well studied nicotine pyridine degradation pathway (34). This proposal can be dismissed because HspB does not convert 2-hydroxypyridine to 2,5-DHP under aerobic conditions. The second hypothesis for the HspB-catalyzed transformation of HSP assumes an initial Baeyer-Villiger reaction to produce an ester intermediate, followed by hydrolysis to the products. This combination of reactions is analogous to that used in the conversion of cyclohexanone to 6-hydroxyhexanoate by cyclohexanone monooxygenase (35), however, the latter reaction also requires a lactonase to hydrolyze the intermediate. For the HspB reaction to catalyze such a Baeyer-Villiger reaction the carbonyl group of HSP would undergo nucleophilic attack by a C(4a)-peroxyflavin, followed by Criegee rearrangement to the ester intermediate (35). The ester intermediate has not been detected in the reactions using His-HspB, and there is no evidence of esterase activity associated with this enzyme. Furthermore, this chemistry does not account for the ability of His-HspB to convert PNP to the quinone. In the third hypothesis, the C(4a)-hydroperoxyflavin reacts with the side chain-substituted carbon of the pyridine ring (Fig. 12). As described in more detail below, this reaction is consistent with all of our experimental results.

HspB, like the substrates of two other pyridine β-hydroxylating enzymes (DHPH and 6HN3M), possesses a C-6 hydroxyl group (Fig. 1A). This observation suggests that the C-6 hydroxyl moiety may participate in the reaction. Indeed, we showed that SP, an intermediate in the pyridine pathway prior to the step catalyzed by HspB and lacking the C-6 hydroxyl group, is not transformed by His-HspB. This result thus points to the possibility of the hydroxyl group being essential for the HspB catalytic reaction. Deprotonation of this hydroxyl group for the three pyridine derivatives leads to quinoid-like compounds with the charge partially delocalized to the carbon opposite to the hydroxyl moiety. Attack of this nucleophilic carbon on the C(4a)-hydroperoxyflavin would generate the C(4a)-hydroxide (which decays by loss of water to generate the oxidized flavin) and an unstable species (intermediate 1 in Fig. 12) that would favor hydrolysis involving a retro-aldol reaction due to the resulting rearomatization.

Support for the above hypothesis is also provided by studies with enzymes acting on non-pyridine, homocyclic compounds. For example, PNP can be transformed into quinone by PspA (Fig. 1B). This transformation likely proceeds by reaction of the
deprotonated substrate with hydroperoxyflavin to yield a quinone-like species with the newly introduced hydroxide and the nitro group on the same carbon; this intermediate decays to the quinone with release of NO2. Of interest, our HPLC results with His-HspB was consistent with the same transformation of PNP to quinone, followed by spontaneous reduction of the quinone to hydroquinone by NADH. Another example of an enzyme using this mechanism is PHB1H, which catalyzes the 1-hydroxylation of the homocyclic analog of 6-hydroxy-nicotinate. This enzyme is proposed to form a hydroperoxyflavin that performs an electrophilic attack on a resonance form of p-hydroxybenzoate with partial anionic charge on the carbon to which the carboxylate is attached; the resulting transient quinone intermediate undergoes decarboxylation accompanied by reorganization to produce its product (Fig. 1B) (36). Finally, the reaction catalyzed by 4HPA1M (Fig. 1B) also has been suggested to involve formation of a quinoid intermediate in which the acetate moiety is located on the same carbon as the newly introduced oxygen atom; in this case the intermediate decays with acetyl group migration (37). These examples support the hypothesis that HspB uses the mechanism shown in Fig. 12 and is mechanistically similar to other group A flavin-dependent monooxygenases.

Several additional comments are noteworthy regarding the proposed HspB reaction shown in Fig. 12. In the reductive half-reaction, the presence of HSP stimulates the rate constant of the NADH-catalyzed reduction reaction by ~150-fold. Such stimulation of the reduction step by bound substrate is commonly found in group A flavin-dependent monooxygenases (38) where it serves as a mechanism to minimize the uncoupled reaction (where NADH is consumed as H2O2 is produced) when substrate is absent. Following reduction of the flavin, the NAD+ is released from the protein, as demonstrated by similar reaction kinetics regardless of the NAD+ concentration (Fig. 10B). Finally, a C4a-(hydro)peroxyflavin intermediate was directly detected by transient spectroscopy of the oxidative half-reaction when using enzyme samples incubated with HSP. The C4a-intermediate is unstable and was only detected within the first few milliseconds of the reaction. The absence of this intermediate for samples lacking HSP suggest the substrate acts to stabilize the C4a-intermediate. This intermediate reacts with HSP, resulting in the formation of products and presumably the C4a-hydroxyflavin, which dehydrates to the oxidized flavin.

The overall reaction catalyzed by HspB is similar to that of many other flavin-dependent monooxygenases: the oxidized enzyme takes up its substrate, NADH binds, hydride transfer takes place to generate the reduced enzyme complex, and NAD+ is released (26, 39). The reduced enzyme complex then activates oxygen to form the C4a-peroxyflavin, which is then protonated, or it directly forms a C4a-hydroperoxyflavin. This species decays to generate hydrogen peroxide in the absence of substrate (as seen for HspB lacking HSP or in the presence of the poor substrate PNP). The binding of substrate stabilizes the C4a-hydroperoxyflavin, thus facilitating further steps in the enzymatic reaction. In the case of HspB, HSP is likely to be activated by an active site residue that forms a hydrogen bond with or deprotonates the C-6-hydroxy group (Fig. 12). This activation provides electron density at C-3 (the side chain-substituted carbon), which then reacts with the C4a-hydroperoxyflavin to yield a 3-hydroxy-substituted species (Intermediate I in Fig. 12). Subsequent addition of water to the ketone (yielding Intermediate 2) accounts for the incorporation of solvent into the product succinate, formed by a retro-aldol reaction (40, 41). Thus, the amino acid sequence and several aspects of its mechanism reveal that HspB belongs to the group A flavin-dependent monooxygenase family, despite having two Rossmann-folds.

Organic synthesis of pyridine derivatives is commonly accompanied by the formation of by-products (42). In contrast, the enzymes involved in microbial metabolism usually have high substrate specificity and favor a single product. Bacterial pyridine hydroxylation is ubiquitous in the environment (11); however, biochemical studies of pyridine hydroxylases have been limited. This mechanistic study of HspB provides useful information that can be applied to the study of other pyridine hydroxylases, thus expanding biotechnology applications in the synthesis of pyridine derivatives.

In conclusion, this study reveals that HspB catalyzes a monooxygenation reaction that results in carbon–carbon cleavage during the transformation of HSP to 2,5-DHP and succinate. The reaction kinetics elucidated the reaction mechanism of HspB and demonstrated a role for HSP in stimulating the reductive half-reaction in catalysis. A C4a-oxygenated reaction intermediate was directly observed and shown to be involved in the hydroxylation reaction. The C4a-hydroperoxyflavin intermediate reacts with the side chain-substituted carbon of the pyridine ring to form a quinone-like intermediate, which is converted into the products through a retro-aldol rearrangement. Elucidation of the biochemical properties of HspB not only supplements our understanding of the pyridine hydroxylases, but also serves as a model for understanding other enzymes involved in the synthesis of pyridine derivatives.

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