Molecular Determinants of Efficient Cobalt-Substituted Hemoprotein Production in *E. coli*

Brian R. Weaver, Lydia J. Perkins, Froylan Omar Fernandez Candelaria, Judith N. Burstyn,* and Andrew R. Buller*

**ABSTRACT:** Exchanging the native iron of heme for other metals yields artificial metalloproteins with new properties for spectroscopic studies and biocatalysis. Recently, we reported a method for the biosynthesis and incorporation of a non-natural metallocofactor, cobalt protoporphyrin IX (CoPPIX), into hemoproteins using the common laboratory strain *Escherichia coli* BL21(DE3). This discovery inspired us to explore the determinants of metal specificity for metallocofactor biosynthesis in *E. coli*. Herein, we report detailed kinetic analysis of the ferrochelatase responsible for metal insertion, *Ec*HemH (*E. coli* ferrochelatase). This enzyme exhibits a small, less than 2-fold preference for Fe$^{2+}$ over the non-native Co$^{2+}$ substrate in vitro. To test how mutations impact *Ec*HemH, we used a surrogate metal specificity screen to identify variants with altered metal insertion preferences. This engineering process led to a variant with an $\sim$30-fold shift in specificity toward Co$^{2+}$. When assayed in vivo, however, the impact of this mutation is small compared to the effects of alteration of the external metal concentrations. These data suggest that incorporation of cobalt into PPIX is enabled by the native promiscuity of *Ec*HemH coupled with BL21’s impaired ability to maintain transition-metal homeostasis. With this knowledge, we generated a method for CoPPIX production in rich media, which yields cobalt-substituted hemoproteins with $>95\%$ cofactor purity and yields comparable to standard expression protocols for the analogous native hemoproteins.

**KEYWORDS:** ferrochelatase, metal homeostasis, multiplexed screening, enzyme kinetics

Proteins bearing unnatural metallocofactors are useful tools to study the metalprotein function, expand the reactivity of natural proteins, and catalyze abiological reactions.$^{1,2}$ In particular, metal-substituted hemoproteins have been explored extensively because they have unique reactivity,$^{3-8}$ may serve as spectroscopic probes,$^{9-12}$ and have unique imaging properties. While there are diverse methods to generate proteins loaded with unnatural metalloporphyrin cofactors both in vivo and in vitro,$^{13-15}$ many of these strategies require exogenous synthesis of the unnatural metalloporphyrin and rely on low-efficiency methods for inserting the cofactor into the protein. We recently reported a method for the de novo biosynthesis of the non-native heme analogue, cobalt protoporphyrin IX (CoPPIX), in *Escherichia coli* BL21(DE3) and its incorporation into hemoproteins.$^{16}$ This fully biosynthetic method streamlined the production of CoPPIX bearing artificial metalloproteins and spurred further inquiry into the determinants of metal specificity in the metalloporphyrin assembly in *E. coli* BL21(DE3).

Nature typically assembles metalloproteins and metallocofactors with an impressive fidelity. Nevertheless, there are reports of improperly metalated heme cofactors occurring in living systems. In humans, zinc protoporphyrin IX (ZnPPIX) is a biomarker for altered iron homeostasis and is implicated in some thalassemias.$^{17}$ Majtan et al. found that *E. coli* BL21-Rosetta2(DE3), when passaged for several days

**Received:** August 7, 2023
**Revised:** October 2, 2023
**Accepted:** October 31, 2023
through iron-poor, cobalt-rich media, adventitiously biosynthesized and incorporated CoPPIX into both endogenous and heterologously expressed hemoproteins. We reported an expansion of this finding by showing that the standard laboratory strain *E. coli* BL21(DE3) innately possesses the ability to biosynthesize and incorporate CoPPIX, without passaging. Addition of an inexpensive cobalt salt to iron-deficient minimal media was sufficient to produce cobalt-substituted hemoproteins from diverse fold families with >95% cobalt loadings. Others similarly found that ZnPPIX can be overproduced by an engineered B-derived strain of *E. coli* under iron-poor, zinc-rich fermentation conditions.20 These reports indicate that heme biosynthesis in *E. coli* may be manipulated toward the production of non-natural metalloporphyrin cofactors. However, these cases of alternative heme metalation in *E. coli* depend on iron-deficient growth conditions to reduce the production of the native heme cofactor. Such conditions severely limit the cell growth and, correspondingly, the titers of expressed cobalt-substituted hemoproteins are low when compared to standard expression of hemoproteins.16 We envisioned that by understanding determinants of cofactor metalation specificity, we might develop a more efficient process for the synthesis and incorporation of the CoPPIX cofactor.

Metalation of tetrapyrrole-derived cofactors (heme, siroheme, cobalamin, and others) is a physiologically irreversible process catalyzed by a diverse class of enzymes called chelatases.21 In *E. coli*, the final step in heme b biosynthesis is incorporation of ferrous iron into PPIX, catalyzed by PPIX ferrochelatase (*E. coli* ferrochelatase, EcHemH) (Figure 1A).

![Figure 1. Reaction of EcHemH with ferrous iron and PPIX to make heme b. Two distinct molecular pathways for metal insertion have been proposed, and the residues responsible for orienting the metal are suggested to contribute. Here, we explore *E. coli* BL21(DE3)'s unusual ability to produce CoPPIX in vivo. To better understand the native metal specificity of EcHemH, we used a purified enzyme to measure the catalytic activity with Fe$^{2+}$, Co$^{2+}$, Ni$^{2+}$, Cu$^{2+}$, and Zn$^{2+}$. We next asked whether a more selective cobalt chelatase might enable efficient production and incorporation of CoPPIX in iron-rich growth media. We used a substrate-multiplexed metal specificity to identify a single-point mutant (L13R) that has a ∼30-fold shift in selectivity for Co$^{2+}$ over Fe$^{2+}$ relative to the parent EcHemH. We analyzed the ability of EcHemH variants to discriminatingly produce CoPPIX over heme in rich growth media and found that, while variants may produce altered specificity profiles in vivo, metabolic factors play the predominant role in determining CoPPIX incorporation into coexpressed hemoproteins. Under sufficiently high cobalt concentrations in rich media and in the absence of a specifically engineered chelatase, BL21(DE3) reproducibly yields greater than 95% cobalt-loaded hemoprotein with titers comparable to those of typical heme protein expressions. Our results provide insight into the mechanism and metal selectivity of EcHemH, inform future engineering efforts for metalloporphyrin biosynthesis, and demonstrate a straightforward and scalable route to cobalt-substituted hemoprotein production in rich media.

### RESULTS

**In Vitro Assessment of EcHemH Metal Promiscuity.**

We began our investigation by probing the kinetic mechanism and metal specificity of EcHemH (UniProt accession: A0A140NEM8). This protein contains a C-terminal His-tag and is overexpressed in *E. coli* BL21(DE3), followed by purification with nickel affinity chromatography yielding approximately 10 mg EcHemH per L culture. While poly-His tags may interfere with the metal binding properties of some enzymes, such constructs for chelatase homologues have been used previously, without complication.17,39–41 Sodium cholate was added to the lysis, purification, and storage buffers, as the enzyme has been shown to require detergents for solubility.42 EcHemH is predicted to be membrane associated due to the presence of a 12-residue segment implicated in membrane interaction.

Using a simple spectroscopic assay, we investigated the ability of EcHemH to catalyze the insertion of Fe$^{2+}$, Co$^{2+}$, Ni$^{2+}$, Zn$^{2+}$, Cu$^{2+}$, Mn$^{2+}$, and Mg$^{2+}$ into PPIX. The catalytic activity of ferrochelatase is well suited to kinetic analysis by electronic-absorption spectroscopy, as the metalation of PPIX imparts unique spectral shifts to the Soret (∼400 nm) and Q bands (∼500–600 nm). We detected product formation for all metals tested, except for Mn$^{2+}$ and Mg$^{2+}$ (Figures 2A,C and S1). The reactivity profile of EcHemH was in good agreement with the reported activity of previously characterized ferrochelatase enzymes.25 To quantify the native iron chelatase activity of EcHemH, we measured initial rates of heme formation spectrophotometrically with variable PPIX and Fe$^{2+}$ concentrations at room temperature (Figures 1B and S2). We fit these data to an ordered sequential mechanism, where PPIX binds first, followed by Fe$^{2+}$ binding, as described by Scheme
Figure 2. EcHemH-catalyzed insertion of native Fe²⁺ and non-native metals into PPIX. (A) Progress spectra of Fe²⁺ insertion into PPIX by EcHemH. The black trace represents the UV–visible spectrum of 7.5 μM PPIX in reaction buffer [100 mM 3-(N-morpholino)propanesulfonic acid, 400 mM NaCl, and 0.2% v/v Tween 80 at pH 7.0] with 100 nM EcHemH. The metalation reaction was initiated at 25 °C by the addition of 10 μM of Fe²⁺. To limit competing oxidation to Fe³⁺, samples were prepared fresh, and initial velocities were taken from the first 30 s of data acquisition. Gray lines indicate absorption spectra taken during the reaction progress, and the pink line represents the absorption spectrum of heme b (FePPIX) at the end of the reaction. (B) Initial rates of EcHemH-catalyzed heme production plotted as a function of the Fe²⁺ concentration. The concentration of PPIX was 5 μM. These reactions were conducted at 25 °C. The solid line represents the best fit of the data using an ordered sequential binding kinetic model, as shown in Figure S2. (C) Progress spectra of Co²⁺ insertion into PPIX by EcHemH. The experimental setup is identical to that of (A), except that 30 nM EcHemH and 10 μM Co²⁺ were used in the reaction. The purple line represents the absorption spectrum of CoPPIX at the end of the reaction. (D) Initial rates of EcHemH-catalyzed CoPPIX production plotted as a function of Co²⁺. Rates were measured with PPIX concentrations ranging from 0.1 to 10 μM PPIX, only three concentrations of which are shown here for clarity. The data were fit globally by using DynaFit 4. Solid lines represent the best fit of the data using a noncompetitive substrate model [see (E)] and correspond to the kinetic parameters shown in Table 1. Additional data and information about the kinetic model used can be found in Figures S3 and S10 of the Supporting Information. (E) Cartoon representation of the proposed noncompetitive substrate inhibition kinetic scheme (see also Scheme S2). (F) Progress curves tracking CuPPIX production over time. Reactions were conducted at 25 °C with approximately 5 μM (gray) or 10 μM (black) PPIX and 100 nM EcHemH and were initiated by the addition of approximately 3.8 μM Cu²⁺. Solid lines represent the best global fit of the data by DynaFit 4 to the noncompetitive substrate inhibition model (Scheme S2) and correspond to the kinetic parameters shown in Table 1.

S1. An equilibrium random-ordered mechanism can also describe these data, which results in identical macroscopic constants as the ordered sequential kinetic model. The resulting fit yielded a $k_{cat}$ of 30 min⁻¹, a $K_M^{Fe}$ of 0.48 μM Fe²⁺, and a $K_M^{PPIX}$ of 1.1 μM PPIX (Table S7). These values compare favorably with previously characterized ferrochelatase enzymes (Table S3).³²

We next sought to test the kinetics of CoPPIX formation by EcHemH. We first measured the initial rates of CoPPIX production at 5 μM PPIX while varying the Co²⁺ concentration. In contrast to the behavior observed with Fe²⁺, we observed a decrease in the rate of CoPPIX production with high concentrations of Co²⁺ (Figure 2D). This substrate inhibition has been observed previously with yeast⁴⁶ and mouse⁵⁵ FECH enzymes, as well as with coproporphyrin(III) ferrochelatase (CpfC) of other species.⁴⁰,⁴¹ To characterize this inhibition further, we measured initial rates of CoPPIX production while varying the concentrations of both Co²⁺ and PPIX (Figure 2D, 3 of 6 PPIX concentrations shown for clarity). The resulting data show that increasing concentrations of PPIX exacerbate the inhibitory effect of Co²⁺. These data mirror observations by Davidson et al. in studies of human HemH with a PPIX analogue, mesoporphyrin IX, and Zn²⁺.²⁴,³⁰

To better understand the nature of Co²⁺ inhibition, we turned to a global analysis of the Co²⁺ initial rate data to identify the simplest kinetic mechanism that recapitulates this complex kinetic behavior. A complete accounting of each kinetic model that we tested is provided in the Supporting Information (Figures S3–S10). Notably, none of the existing kinetic models for chelatases in the literature fit the data.⁴⁰,⁵² On the basis of this analysis, we propose an EcHemH kinetic mechanism that includes two kinetically distinct metal binding modes, only one of which is kinetically productive (Figure 2E). The productive pathway is initiated by PPIX binding. Co²⁺ can then bind in either a productive mode, leading to metal insertion, or a nonproductive mode corresponding to noncompetitive inhibition. In this model, metal can also bind prior to PPIX, but it leads to an inhibitory ternary complex. Together, these model features accurately recapitulate key kinetic observations. At low Co²⁺ concentrations, metal binding in the catalytically productive mode (i.e., after PPIX binding) dominates, and the initial velocities increase as the concentration of PPIX increases. However, at high PPIX concentrations, increasing Co²⁺ concentrations slow the metalation because noncompetitive binding occurs, leading to pronounced substrate inhibition (Figure 2E). We note that Fe²⁺ insertion does not show substrate inhibition within the range of concentrations tested here nor has substrate inhibition with Fe²⁺ been observed with other ferrochelatases. However, substrate inhibition was previously observed for the Cu²⁺
insertion into PPIX by the yeast homologue. These studies showed distinctive “S”-shaped progress curves wherein reactions accelerate during a time course due to relief of substrate inhibition. We tracked CuPPIX formation by EcHemH spectroscopically with three different PPIX and Cu\(^{2+}\) concentrations and observed the same S-shape in the progress curves (Figure 2F). We fit these progress curve data to the noncompetitive binding model (Figure 2E) and found that the data were well recapitulated, including the characteristic S-feature. We compared the macroscopic kinetic parameters for insertion of Fe\(^{2+}\), Co\(^{2+}\), and Cu\(^{2+}\), which affirm the remarkable promiscuity of the enzyme (Table 1).

Table 1. Kinetic Profile of EcHemH Activity with Various Metals

<table>
<thead>
<tr>
<th>substrate</th>
<th>(k_{cat}) (min(^{-1}))</th>
<th>(K_m) (metal) ((\mu)M)</th>
<th>(k_{cat}/K_m) ((\mu)M(^{-1}) min(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fe(^{2+})</td>
<td>30</td>
<td>0.48</td>
<td>63</td>
</tr>
<tr>
<td>Co(^{2+})</td>
<td>54</td>
<td>1.1</td>
<td>48</td>
</tr>
<tr>
<td>Cu(^{2+})</td>
<td>34</td>
<td>0.94</td>
<td>36</td>
</tr>
</tbody>
</table>

Apparent macroscopic kinetic parameters for Fe\(^{2+}\), Co\(^{2+}\), and Cu\(^{2+}\) are derived from fitting the data to the appropriate kinetic model. Parameters were not well determined for Zn\(^{2+}\) activity with Co\(^{2+}\)Structure. We hypothesized that promiscuous diversity of transition metals, non-native metals and the relative efficiency of metals highlights the prevalence of substrate inhibition with different metals engage in distinct kinetic mechanisms. These studies stood, although examples of variants with altered specificity have been found.

We used a model generated with AlphaFold to compare structural features of EcHemH to other, structurally characterized homologues (Figure 3A). Alignment of EcHemH with the PPIX-bound HsFECH structure (PDB ID: 2HRE, 27% sequence identity) suggested how EcHemH might interact with the porphyrin substrate. The main structural difference between HsFECH and EcHemH is the presence of an iron–sulfur cluster at the C-terminus of HsFECH. Despite the low overall sequence identity, many active site residues appear to be highly conserved. Distinctive residues in EcHemH are Leu13, Phe31, and Cys273, which correspond to Met76, Leu92, and His341 in HsFECH, respectively (Figure 3B). The differences at Leu13 (E. coli) and Met76 (human) are noteworthy because these residues lie directly below the porphyrin substrate, and Met76 has been implicated in metal specificity for HsFECH.

To gain further insight into sequence conservation across the enzyme family, we generated a sequence alignment of 5026 HemH homologues and visualized active site conservation with the PPIX-bound HsFECH structure (PDB ID: 2HRE, 27% sequence identity) suggested how EcHemH might interact with the porphyrin substrate. The main structural difference between HsFECH and EcHemH is the presence of an iron–sulfur cluster at the C-terminus of HsFECH. Despite the low overall sequence identity, many active site residues appear to be highly conserved. Distinctive residues in EcHemH are Leu13, Phe31, and Cys273, which correspond to Met76, Leu92, and His341 in HsFECH, respectively (Figure 3B). The differences at Leu13 (E. coli) and Met76 (human) are noteworthy because these residues lie directly below the porphyrin substrate, and Met76 has been implicated in metal specificity for HsFECH.

To gain further insight into sequence conservation across the enzyme family, we generated a sequence alignment of 5026 HemH homologues and visualized active site conservation.
with logo plots shown in Figures S11–S20. Several striking patterns were observed. For example, His194, Trp242, Phe269, and Glu275 (E. coli numbering) are >99% conserved, suggesting that these residues are especially important for ferrochelatase function. Additionally, the positions corresponding to Leu13 and Phe31 are almost exclusively occupied by hydrophobic residues. This analysis provides evolutionary context for choosing which residues might be most amendable to mutation. We screened site-saturation mutagenesis (SSM) libraries at 10 active site residues (Figure 3A, labeled residues). Based on our structural model, we hypothesized that these residues might impart changes in metal specificity either by altering metal chelation or by altering the extent and nature of porphyrin distortion.

**Altering Metal Specificity of EcHemH through SSM.** Substrate specificity can be a challenging feature to optimize through engineering because traditional approaches typically monitor the activity on a single, model substrate as a proxy for the overall enzyme performance. These assays cannot distinguish between enzymes that have a higher expression or higher catalytic activity. For the study here, we were more interested in the relative activity of variants on different metals in competition than in the overall catalytic prowess. Therefore, we developed a substrate-multiplexed screen (SUMS) with a mixture of metal ions that provided direct information on enzyme specificity in a single experiment. The SUMS approach, as applied to protein engineering, has been shown to facilitate the discovery of enzyme variants with altered specificity and identification of distal residues that impact catalysis.38–50

While the relative activity of Fe²⁺ versus Co²⁺ was our principal interest, we found that the specificity between these two metals was exceedingly difficult to monitor in cell lysates. At least two factors confounded analysis. First, Fe²⁺ quickly oxidizes in the lysate to Fe³⁺, which is not a substrate for EcHemH.32 Second, heme b is catabolized in the cell lysate, leading to inconsistent ratios relative to CoPPIX. To work around these challenges, we hypothesized that screening on a mixture of other, non-native metals would reveal residues that provide the most significant changes to metal specificity relative to the parent. These mutations could subsequently be analyzed to gain insight into their Fe²⁺ specificity using an alternative screening method.

Cell lysates containing EcHemH variants were added to a mixture of Co²⁺, Ni²⁺, and Cu²⁺ at a relatively high concentration (1 mM total M²⁺) in the presence of 10 μM PPIX. The relative distribution of the porphyrin products was directly measured using ultrapressure liquid chromatography (UPLC), and the concentration of each metal was adjusted such that the parent EcHemH produced approximately equal signals for CoPPIX, NiPPIX, and CuPPIX (see Supporting Information). The resulting data from screening 10 SSM libraries are compiled in the Supporting Information (Figures S11–S20). We note that the uncertainties associated with screening preclude quantitative analysis on the absolute abundance of each product.

While site-saturation studies of HemH homologues have not been previously reported, the data here were in good general agreement with the detailed analysis performed on point mutants of homologous enzymes. For example, the EcHemH H194X library yielded no active variants (Figure S10). Studies of corresponding variants of FECH (H263C and H263A HsFECH and H235L ScFECH) indicated these variants were inactive or had only trace activity. Indeed, H194 is nearly 100% conserved among HemH/FECH homologues, consistent with an essential catalytic role.

To directly compare specificity shifts among variants from different libraries, we rescreened in parallel a subset of variants that showed the largest shifts in specificity in the initial screen. While the trends in activity were generally reproducible, several variants had different specificities in this follow-up assessment when compared to the initial screens (e.g., L13R, compare Figures 3 and S11). From the rescreen data, we identified several variants with altered metal specificity compared to the parent EcHemH that we chose for further validation in comparison against Fe²⁺ (Figure 3B).

**Assessing EcHemH Variant Specificity for Fe²⁺ versus Co²⁺.** We next sought to investigate how variants with in vitro changes in metal specificity altered metalloporphyrin incorporation into hemoproteins in vivo. Cells harboring overexpressed EcHemH variants were grown in rich media supplemented with a 4:1 ratio of exogenous Co²⁺ to Fe²⁺, such that the bioavailable metal pool was consistent and well defined. To directly assess the effect of chelatase identity on hemoprotein loading, we coexpressed EcHemH with the hemoprotein, dye-decolorizing peroxidase (DyP, UniProt Accession E3G914). DyP binds free PPIX, CoPPIX, and heme b promiscuously. Following expression, DyP was purified by nickel-affinity chromatography, and the relative Co⁵⁺ and Fe⁵⁺ contents of the protein samples were measured using inductively coupled plasma mass spectrometry (ICP–MS). This analysis showed that several variants incorporated more CoPPIX relative to the parent EcHemH, including L13R, L13H, R57Q, and E275D (Figures 4A and S21). Many factors, such as the enzyme expression level, may impact metalation outcomes. Indeed, these in vivo experiments relied on the overexpression of EcHemH and its variants to circumvent the native EcHemH regulation and deliver high concentrations of enzyme. However, there remained the possibility that high titers of EcHemH may influence metalloporphyrin production relative to that of the native BL21(DE3) system that motivated this study. We therefore expressed and purified DyP without overexpressing EcHemH and assessed its metal content. This experiment was intended as a negative control but instead showed that the CoPPIX content of DyP without overexpression of EcHemH was the same or higher than any other conditions tested (Figure S21). This result was the first clue that, to our surprise, ferrochelatase specificity may be a minor contributor to in vivo metalation outcomes. To rigorously test this new hypothesis, we validated a single variant with altered specificity and explored its impact (or lack thereof) on in vivo PPIX metalation.

**Testing the Effects of Altered Chelatase Specificity.** We selected EcHemH L13R to assess the change in metal specificity relative to the parent. Rather than determining complex kinetic parameters with this enzyme, we opted for a direct measurement of the change in selectivity by assaying the metalation outcome when Co²⁺ and Fe²⁺ are in direct competition. Excess ascorbate was added to maintain the ferrous oxidation state, and product formation was analyzed by UPLC. This analysis revealed that the L13R mutation imparts a ~30-fold shift in selectivity for Co²⁺ relative to the parent under these conditions (Figure 4B).

We next asked whether coexpression of the cobalt-selective EcHemH variant L13R had any significant impact on CoPPIX versus heme incorporation in rich media. We compared the
**Figure 4.** Competition experiments for Co$^{2+}$ and Fe$^{2+}$ incorporation into PPIX by the parent E. coli EcHemH and the L13R EcHemH variant. (A) Distribution of MPPiX products obtained from coexpression of the parent and L13R EcHemH with dye-decolorizing peroxidase (DyP) with a defined mixture of 50 μM Fe$^{2+}$ and 100 μM Co$^{2+}$ added to the growth media. DyP was purified by Ni-affinity chromatography and digested in nitric acid prior to analysis by ICP-MS. Data are the average of triplicate ICP-MS measurements. (B) In vitro measurement of Co$^{2+}$ vs Fe$^{2+}$ incorporation into PPIX by purified parent and L13R EcHemH enzymes. Reactions were conducted in 100 mM Tris–HCl buffer with 400 mM NaCl and 0.2% Tween 80. The concentration of enzyme added was 250 nM, and the concentration of PPIX was 5.0 μM. Reactions were initiated with the addition of 50 μM each of Fe$^{2+}$ and Co$^{2+}$ in 0.5 mM ascorbate. After 20 min, reactions were quenched with acid and extracted into an organic solvent. The relative concentrations of porphyrin products were analyzed by UPLC using the absorbances at 400 nm (FePPIX and PPIX) and 423 nm (CoPPIX). Error bars represent the standard deviation of the quadruplicate UPLC measurements.

**Figure 5.** Effect of HemH expression, cobalt concentration, and E. coli expression strain on the distribution of CoPPIX- and heme-loaded DyP (A) metal content analysis of DyP by ICP-MS. Proteins were expressed in BL21(DE3) in the absence of a coexpression vector, with parent E. coli EcHemH and with EcHemH L13R. CoCl$_2$ (1 mM) and 250 μM δ-aminolaevulinic acid were added at the time of induction. Dots represent biological replicates. (B) ICP-MS measurements of DyP expressed in BL21(DE3) with various amounts of cobalt. Cobalt was added when OD$_{600}$ reached 0.2–0.3, prior to induction with 7-arabinose. Measurements were made in triplicate, and error bars represent standard deviation. (C) ICP-MS measurements of DyP expressed in JM109(DE3) with parent and L13R EcHemH coexpression and in the absence of chelatase coexpression. Expression conditions were identical to that of Figure 6A. Measurements were made in triplicate, and error bars represent standard deviations.
Validation of a Method for Cobalt-Substituted Hemoproteins in Rich Media. With this new perspective on the determinants of CoPPIX production, we set out to generate a robust method for the production of cobalt-substituted hemoprotein in rich media. We found that the timing of addition of 1 mM CoCl₂ to the media had no significant effect on the metalloporphyrin content and yield of holoprotein (Table S5). Consequently, we converged on a protocol where metal is added at the time of induction, along with δALA, which was convenient and effective. With this method, we were able to obtain CoDyP at a titer of 43 mg protein per L cell culture, and the resulting proteins were >95% cobalt loaded relative to iron. This yield represents a nearly 10-fold improvement in protein titer over our previous method in minimal media and gives similar titers to the media had no significant effect on the metalloporphyrin content and yield of holoprotein (Table S5). Consequently, we converged on a protocol where metal is added at the time of induction, along with δALA, which was convenient and effective. With this method, we were able to obtain CoDyP at a titer of 43 mg protein per L cell culture, and the resulting proteins were >95% cobalt loaded relative to iron. This yield represents a nearly 10-fold improvement in protein titer over our previous method in minimal media and gives similar titers to the expression of the native heme-loaded protein (Figure 6).

Lastly, we tested the efficiency of cobalt-substituted hemoprotein production with a different protein scaffold, the P450 enzyme CYP119 from Sulfolobus acidocaldarius, under pET22b (IPTG-inducible) and pBAD (arabinose-inducible) expression systems. From 1 L of cell cultures in rich media, we obtained 2 and 43 mg of ~95% CoPPIX-loaded hemoprotein from the pET22b and pBAD systems, respectively. This expression compares favorably with that of the analogous heme proteins, which, in our hands, were obtained at 3 and 40 mg per L culture. Furthermore, this method requires virtually no additional steps or genetic manipulations relative to that of the canonical heme protein, aside from the addition of an inexpensive cobalt salt (CoCl₂) at the time of induction.

DISCUSSION

We performed steady-state kinetic characterization of EcHemH with a variety of metals and found that this enzyme has a notable substrate inhibition with Co²⁺. One molecular interpretation consistent with this phenomenon is metal-ion binding at two different sites, which has been proposed for other ferrochelatases. In this kinetic model (Figure 2E), the binding mode that is populated when no PPIX is present is kinetically off-pathway and inhibitory. Structural analysis of the S. cerevisiae (PDB: 1L8X) and Bacillus subtilis (PDB: 3M4Z) chelatases exhibited metal-ion binding to Glu275 and His194 (E. coli numbering) in the absence of porphyrin.

We hypothesize that these two residues may comprise the inhibitory binding site, which sits “on top” of the porphyrin when the hydrophobic binding interface is oriented “below” (Figure S23). This combination of kinetic and structural evidence suggests that metalation occurs from the bottom face of the porphyrin, as has been proposed for the human FECH enzyme. This region of protein contains Leu13, where we found that the mutation strongly influenced metal specificity. In this scenario, the metal is desolvated as it binds to the PPIX pyrrole nitrogens in the hydrophobic enzyme environment. A base is required to deprotonate two pyrrole rings, and steric considerations demand that these protons are removed from the opposite face of from which metal insertion occurs. In EcHemH, His194 is positioned to play the role of a general base, a hypothesis corroborated by the absence of any chelatase activity in the H194X SSM library.

Our initial hypothesis was that a low level of chelatase promiscuity was the main enabler of adventitious CoPPIX production in BL21(DE3). Noncompetitive substrate inhibition decreases the overall rate of enzyme catalysis when two metals are placed in competition but does not otherwise affect the distribution of products. This inhibition notwithstanding, activity on Fe²⁺ and Co²⁺ was generally similar, indicating that EcHemH is an effective cobalt chelatase. To test the effect of chelatase metal specificity on the production of CoPPIX versus heme in BL21(DE3), we undertook an engineering campaign to improve the Co²⁺ selectivity of native BL21(DE3) ferrochelatase. The enzyme was surprisingly tolerant to mutation, as SSM at seven of ten active site residues yielded active chelatase variants. By screening on a mixture of metal substrates, we were able to probe the influence of residues on metal selectivity in a fashion that is decoupled from overall protein activity. Mutations throughout the active site have marked effects on metal specificity, indicating that complex and synergistic interactions with PPIX, metal, and active site residues all influence metal insertion. These observations align with previous studies that link porphyrin distortions in the active site to metal selectivity. We identified a mutation, L13R, that causes a 30-fold shift in specificity toward cobalt insertion. The discovery of this variant enabled us to explore how the expression of this cobalt-favored chelatase affected the distribution of MPPIX products generated in vivo.

Notably—and contrary to our initial hypothesis—we found that the metal specificity of an overexpressed chelatase plays only a minor role in determining the distribution of metalated porphyrins in BL21(DE3). Large changes in Co²⁺ versus Fe²⁺...
Metabolic homeostasis is a key factor in determining the distribution of metalated porphyrin products in E. coli. BL21-derived strains have been shown to lack the entirety of the \textit{rcn} operon,\textsuperscript{57,59} which is responsible for Co\textsuperscript{2+} and Ni\textsuperscript{2+} efflux.\textsuperscript{60,61} We hypothesize that the success of this cobalt-substituted hemoprotein expression method is dominated by the inability of \textit{E. coli} BL21(DE3) to efflux cobalt. Instead, a high concentration of cobalt in the growth media leads to high cytosolic cobalt concentrations and, as a result, efficient production of CoPPIX by natively promiscuous chelatase. Consistent with this hypothesis, the K12-derived strain JM109(DE3), which contains a functional \textit{rcn} operon, was far less effective than BL21(DE3) in the production of CoPPIX. However, there are many additional differences between BL21- and K12-derived strains of \textit{E. coli}, including changes in central carbon metabolism.\textsuperscript{62}

There may be additional differences in CoPPIX breakdown that influence how much of the cofactor accumulates in different strains. Further studies are required to elucidate the interplay between these diverse factors. For example, in pathogenic \textit{E. coli}, heme is degraded by the heme oxygenase, ChuS (UniProt accession: A0A271QSA5), which is not present in BL21-derived strains.\textsuperscript{63,64} These enzymes catalyze oxidative ring opening of the iron porphyrin cofactor, yielding the linear tetrapyrrole biliverdin \textit{Iα}. These transformations proceed through an iron-oxo intermediate and are inhibited by non-native metalloporphyrins, such as CoPPIX.\textsuperscript{65,66} Heme catabolism in nonpathogenic \textit{E. coli}, such as BL21, is not as well understood.\textsuperscript{57,67} It is possible that \textit{E. coli} BL21(DE3) harbors a yet uncharacterized heme-degrading enzyme or that our and others’ observations of heme loss in cell lysates are due to an alternative, nonenzymatic process. Regardless, native heme catabolic pathways may be specific to heme, allowing CoPPIX to accumulate under expression conditions while excess heme is quickly degraded.

\section*{CONCLUSIONS}

Hemoproteins comprise a diverse family of widely studied and used biocatalysts. Often, BL21(DE3) \textit{E. coli} is the organism used to produce these hemoproteins—and the cofactors they bear—at high titers. By studying the metalation and incorporation of a non-native heme analogue, CoPPIX, we provide insight into how biological metalation is controlled in this ubiquitous model organism and guide future campaigns to engineer metalloc cofactor biosynthesis. Metalloporphyrin distribution may be sensitive to many factors, including chelatase specificity, environmental metal concentrations, metal homeostasis machinery, and cofactor catabolism. In nature, these factors work in concert to ensure the proper metalation of heme. In contrast, laboratory expression conditions can redirect this machinery toward efficient biosynthesis of CoPPIX. We hypothesize that this process proceeds according to the following paradigm: \textit{E. coli} BL21(DE3) does not contain the otherwise common \textit{rcn} operon for efflux of transition metals. These cells grow as normal in rich media and quickly scavenge all available Fe\textsuperscript{2+}, much of which is used by EcHemH to produce heme and support cellular respiration. Once cultures are grown to the mid-log phase and a high concentration of cobalt is added, the cobalt is imported and it accumulates intracellularly. Consequently, there is a large bioavailable pool of Co\textsuperscript{2+} with minimal Fe\textsuperscript{2+} remaining. The native chelatase, EcHemH, has promiscuous activity with this abundant, bioavailable Co\textsuperscript{2+} and produces CoPPIX. When a hemoprotein is contemporaneously overproduced, the CoPPIX is loaded into these proteins as a non-natural cofactor, lessening the toxic effects of both CoPPIX and Co\textsuperscript{2+}.\textsuperscript{15} Free metalloc cofactors may be subject to degradation by heme catabolic pathways, to which the iron cofactor is most susceptible. The net result is an efficient heterologous expression of metal-substituted hemoproteins with the CoPPIX cofactor.

The biosynthesis and incorporation of artificial cofactors are long-standing and alluring challenges in the fields of synthetic biology and biocatalysis. New-to-nature cofactors may imbue enzymes with alternate modes of reactivity, but because these enzymes are difficult and tedious to produce, explorations of their potential activities are rare. The enantioselective carbene and nitrene transfer reactivity of metal-substituted hemoproteins, in particular, has led to the development of several strategies for the incorporation of prefabricated metalloporphyrins into hemoproteins.\textsuperscript{69} The production of these artificial metalloenzymes remains less efficient than that of the native hemoprotein. This report has described a fully biosynthetic and efficient method for the production and incorporation of the artificial cofactor CoPPIX into hemoproteins. This method operates in rich media and thus enables the further development of these proteins as potential biocatalysts.

\section*{ASSOCIATED CONTENT}

\subsection*{Supporting Information}

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acssynbio.3c00481.

Detailed experimental procedures, protein and DNA sequences, LC/MS spectra, time course spectra, initial velocities and kinetic model, progress curves and kinetic model, metal SUMS data from site saturation libraries and logo plots, in vivo selectivity of Co\textsuperscript{2+} vs Fe\textsuperscript{2+} of \textit{EcHemH} variants, structural analysis of HemH homologues, proposed kinetic mechanism for \textit{EcHemH} activity, and optimized method for expression of Co-substituted hemoproteins in rich media (PDF)

\section*{AUTHOR INFORMATION}

\subsection*{Corresponding Authors}

Judith N. Burstyn — Department of Chemistry, University of Wisconsin—Madison, Madison, Wisconsin 53706, United States; Email: burstyn@chem.wisc.edu

Andrew R. Buller — Department of Chemistry, University of Wisconsin—Madison, Madison, Wisconsin 53706, United States; orcid.org/0000-0002-9635-4844; Email: arbuller@wisc.edu

\subsection*{Authors}

Brian R. Weaver — Department of Chemistry, University of Wisconsin—Madison, Madison, Wisconsin 53706, United States; orcid.org/0000-0001-6991-014X

Lydia J. Perkins — Department of Chemistry, University of Wisconsin—Madison, Madison, Wisconsin 53706, United States
Froilan Omar Fernandez Candelaria — Department of Chemistry, University of Wisconsin—Madison, Madison, Wisconsin 53706, United States

Complete contact information is available at: https://pubs.acs.org/10.1021/acssynbio.3c00481

Author Contributions

Funding
This work was supported by the Office of the Vice Chancellor for Research and Graduate Education at the University of Wisconsin—Madison with funding from the Wisconsin Alumni Research Foundation and an NSF CAREER award to A.R.B.

Notes
The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS
We thank members of the Brunold, Buller, and Burstyn groups for fruitful discussions of this research.

■ ABBREVIATIONS

PPIX, protoporphyrin IX
CoPPIX, cobalt protoporphyrin IX
EchHemH, E. coli ferrochelatase
DyP, dye decolorizing peroxidase
SUMS, substrate multiplexed screening

■ REFERENCES


