

Cobalt-substituted hemoprotein expression

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Contents

1. Introduction	2
2. Materials	4
2.1 Reagents and chemicals	4
2.2 Equipment	5
3. Methods	5
3.1 Preparation of stock solution	5
3.2 Transformation protocol for hemoprotein encoding genes	6
3.3 Expression of cobalt-substituted hemoproteins	7
3.4 Purification of cobalt-substituted protein	9
4. Characterization of cobalt-substituted hemoproteins	10
4.1 Measuring concentration of cobalt-substituted hemoproteins	10
4.2 Quantification of cobalt incorporation into hemoproteins	12
4.3 Spectroscopic analysis of cobalt-substituted hemoproteins	16
5. Conclusions	18
Declaration of AI and AI-assisted technologies in the writing process	20
Acknowledgements	20
References	20

Abstract

Substituting the native metal of metalloenzymes can significantly alter the enzymes' reactivity and spectroscopic properties. Cobalt is especially attractive as a substitute for the native iron center in hemoproteins, as it generates metal variants with complementary spectroscopic properties and could enable new modes of reactivity. Here, we describe a detailed protocol for the biosynthesis and incorporation of cobalt protoporphyrin IX (CoPP_{IX}) into hemoproteins, replacing the native heme *b* cofactor during expression in the common laboratory strain *Escherichia coli* BL21(DE3). This protocol is described using the model hemoprotein *Physeter macrocephalus* (sperm whale) myoglobin. Because of cobalt's unique electronic and geometric properties, cobalt-substituted hemoproteins offer a valuable handle for spectroscopic characterization and structural studies. We describe analytic methods of assessing cofactor identity and purity, including electronic absorption spectroscopy, liquid-chromatography/mass-spectrometry, inductively coupled plasma-mass spectrometry, and

electron paramagnetic resonance spectroscopy. This method for generating artificial metalloenzymes is effective, easy to implement, and can produce useful quantities of Co-substituted hemoproteins.



1. Introduction

Engineered artificial metalloenzymes have emerged as promising biocatalysts due to their chemical versatility and tunable selectivity (Davis & Ward, 2019). Ideally, abiological transition-metal substitution in metalloproteins can merge the beneficial properties of enzymes with the broad reactivity of synthetic transition-metal catalysts (Wittwer et al., 2021). Such artificial metalloenzymes enable powerful strategies to dissect structure-function relationships, probe electronic properties, and manipulate reactivity. Cobalt protoporphyrin IX (CoPPiX) is a compelling substitute for heme *b* due to its similar coordination environment, rich spectroscopic features, and broad reactivity demonstrated in small-molecule catalysis (Bruha & Kincaid, 1988; Gupta et al., 2023; Hoffman & Petering, 1970; Ikeda-Saito et al., 1978, 1978; Maret & Vallee, 1993; Smith et al., 2011). Since the initial reports of cobalt-substituted enzymes in the 1970s (Hoffman & Petering, 1970; Wang et al., 1977), their versatility and synthetic utility have steadily expanded, establishing them as valuable spectroscopic analogs and unique biocatalysts (Badarau et al., 2007; Li et al., 2024; Perkins et al., 2024; Sreenilayam et al., 2017; Tu & Silverman, 1985). These studies have demonstrated a demand for a simpler, cost-effective, and generalizable method to obtain cobalt-substituted hemoproteins.

Previous methods to acquire artificial metalloenzymes have contended with labor-intensive cofactor synthesis, inconsistent metal incorporation, and low protein yields (Davis & Ward, 2019; Dydio et al., 2016; Letondor et al., 2005; Nam et al., 2023). One strategy for generating metal-substituted metalloproteins involves removal of the native metal center with a chelator, followed by insertion of a non-native metal. However, this approach does not work with hemoproteins due to the strong binding affinity between the metal and the porphyrin ring. A variety of approaches have been developed to acquire metal-substituted hemoproteins (Majtan et al., 2011; Theorell, 1945; Woodward et al., 2007). For example, researchers have expressed the apo-protein and subsequently added the non-native metalloporphyrin into the cell lysate. The effectiveness of this approach is limited by the low yields associated with apo-protein expression, as many hemoproteins are sparingly

stable in the absence of the cofactor (Dydia et al., 2016; Kawakami et al., 2012). This method further requires a potentially labor-intensive synthesis of the non-native metal-cofactor, which can pose limitations in terms of cost and scalability. Efforts for in vivo incorporation of non-native metal cofactors have been pursued using specialized *E. coli* strains engineered to uptake non-native metalloporphyrins from the growth medium and incorporate them into hemoproteins during expression. For example, an engineered BL21(DE3)-based strain harboring a plasmid encoding the heme transporter ChuA enables the import of exogenous heme analogs (Majtan et al., 2011; Moore et al., 2018; Sreenilayam et al., 2017). However, this method is susceptible to iron contamination, necessitating the use of iron-limited minimal media for cell growth. These limitations have stymied efforts to utilize cobalt-substituted hemoprotein biocatalysts on larger preparative scales (Davis & Ward, 2019).

In this chapter, we describe a reproducible method for the biosynthesis and incorporation of cobalt protoporphyrin IX into proteins that natively bind the heme *b* cofactor using the common laboratory strain, *E. coli* BL21(DE3) cells. The workflow includes adding a cobalt salt and the heme precursor, δ -aminolevulinic acid (δ ALA), to cell cultures at induction, which generates appreciable yields of Co-substituted proteins (Fig. 1). Using this streamlined process, we achieved > 95 % cobalt cofactor purity even in iron-containing rich media (Perkins et al., 2021; Weaver et al., 2023). Several features contribute to the effectiveness of this process. First, and perhaps most significant, the BL21(DE3) cell line lacks the *rcn* operon, which is responsible for regulating cobalt flux and export (Foster et al., 2022). Without this efflux system, cobalt can accumulate intracellularly.

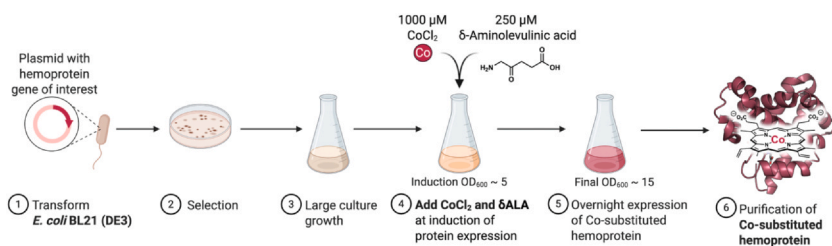


Fig. 1 General workflow of *de novo* biosynthesis of cobalt-substituted hemoproteins. *E. coli* BL21(DE3) cells are transformed with a plasmid encoding the hemoprotein of interest. A single colony is used to inoculate media, and the culture is grown to an OD₆₀₀ of ~5 before induction with 500 μM IPTG, 1000 μM CoCl₂, and 250 μM δ -ALA to produce cobalt-substituted hemoproteins.

Labile cobalt is toxic to cells through multiple mechanisms, and this toxicity is partially mitigated through sequestration into CoPPIX. Second, incorporation of Co^{2+} into protoporphyrin IX (PPIX) is catalyzed by the *E. coli* ferrochelatase, *EcHemH*. This reaction is natively promiscuous and inserts Co^{2+} into PPIX with only a modest 2-fold preference for Fe^{2+} (Weaver et al., 2023). In the protocol presented here, Co^{2+} addition and hemoprotein induction occur at high optical densities, when we hypothesize the bioavailable Fe^{2+} concentration is low. Together, cells tolerate high concentrations of exogenous cobalt during overexpression of a hemoprotein, which is the driving force to effectively generate cobalt-substituted artificial metalloproteins. This cobalt-substitution method has been applied to proteins containing the heme *b* cofactor; however, its application to other types of heme-dependent proteins, such as those containing heme *a* or heme *c*, has not yet been demonstrated. These Co-substituted proteins can be characterized by a variety of spectroscopic techniques and used to expand the native reactivity of hemoproteins for biocatalytic purposes.



2. Materials

The protocol provided here describes the expression, isolation, and spectroscopic characterization of cobalt-substituted myoglobin. This protein contains a C-6-His-tagged variant from a pET-21(+) expression vector. This procedure has been used extensively with a variety of proteins that natively bind heme *b*.

2.1 Reagents and chemicals

- *E. coli* BL21(DE3) expression strain
- Expression plasmid encoding hemoprotein of interest (e.g. pET-21(+))
- $\text{CoCl}_2 \cdot \text{hexahydrate}$
- δ -aminolevulinic acid (δ ALA)
- Terrific Broth (TB)
- M9 minimal medium components
- IPTG (1.0 M stock solution)
- Lysozyme
- DNase I
- Cobalt protoporphyrin IX (CoPPIX) chloride
- Dimethyl sulfoxide (DMSO), HPLC grade

- Ni-NTA resin for affinity purification
- Buffers for lysis and chromatography

2.2 Equipment

- Shaking incubator
- Centrifuge with 4 °C capability
- Sonicator
- Hot water bath
- Chromatography system (e.g., ÄKTA or gravity column)
- UV-vis spectrophotometer
- UPLC-MS
- ICP-MS
- EPR instrument



3. Methods

3.1 Preparation of stock solution

3.1.1 Terrific broth media

1. For 1 L of media, dissolve 47.6 g of terrific broth in 1 L of deionized water.
2. Add 4 mL of glycerol.
3. Autoclave media prior to use.

3.1.2 M9* minimal media

Supplemented M9* minimal media is an adaptation of the recipe from Majtan et al. ([Majtan et al., 2011](#)).

1. Prepare a stock solution of 10x M9 salt solution with 75.2 g/L $\text{Na}_2\text{H}_2\text{PO}_4$, 30 g/L KH_2PO_4 , 5 g/L NaCl, and 5 g/L NH_4Cl in Milli-Q water.
 - a. pH adjust the solution to 7.0 with HCl or NaOH.
 - b. Autoclave the solution.
2. Prepare separate stock solutions of 1 M CaCl_2 and 1 M MgSO_4 and autoclave.
 - a. For 1 M CaCl_2 stock solution, dissolve 14.70 g $\text{CaCl}_2 \cdot 7\text{H}_2\text{O}$ in 100 mL of Milli-Q water.
 - b. For 1 M MgSO_4 stock solution, dissolve 24.65 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ in 100 mL in Milli-Q water.
3. Prepare a stock of 20 % (w/v) glucose by dissolving 200 g/L of glucose in Milli-Q water and sterile filter.

4. Prepare a CAA supplement stock by dissolving 20 g of cas amino acids (0.4 g/mL) and 50 mg of thiamin-HCl (1 mg/mL) in 50 mL of Milli-Q water and sterile filter.
5. To assemble 1 L of M9 * media for culture growth, add 100 mL of 10x M9 salt solution (33.7 mM K_2HPO_4 , 22.0 mM KH_2PO_4 , 8.55 mM NaCl, 9.35 mM NH_4Cl), 10 mL of CAA supplement stock, 10 mL of 20 % (w/v) glucose (0.4 %), 1 mL of 1 M $MgSO_4$ stock (1 mM final), and 200 μ L of 1.0 M $CaCl_2$ stock (0.2 mM final) to 879 mL of autoclaved Milli-Q water.

3.1.3 Storage buffer

1. For 1 L of 100 mM potassium phosphate buffer (pH 7.0) and 100 mM NaCl, add 9.343 g of potassium phosphate dibasic, 6.309 g of potassium phosphate monobasic, and 5.84 g of NaCl to 800 mL of deionized water.
2. pH adjust the solution to 7.0 using either HCl or NaOH.
3. Add deionized water until the total volume is 1 L.

Note: The pH of the storage buffer is hemoprotein dependent. For example, CYP119 from *Sulfolobus acidocaldarius* is best stored at pH 6.0 for optimal catalytic activity. Additionally, Milli-Q water is best suited to prepare buffers for sensitive spectroscopic samples to minimize potential contamination.

3.1.4 CoPPIX chloride stock solution in DMSO

1. Dissolve CoPPIX chloride in DMSO to a final concentration of 2 mM.
2. Filter sterilize (0.22 μ m) and store aliquots at $-20^\circ C$, protected from light.
3. CoPPIX solutions are stable for several months under these conditions.

Note: CoPPIX is poorly soluble in aqueous buffers and should be added as a DMSO solution.

3.2 Transformation protocol for hemoprotein encoding genes

1. Incubate *Escherichia coli* BL21(DE3) chemically competent cells with 1–10 ng of pET-21(+) plasmid encoding hemoprotein of interest for 30 min. Cells should be stored at $-80^\circ C$ prior to use and thawed on ice for 10 min.
2. Following standard protocol, heat shock cell mixture for 10 s at $42^\circ C$ directly followed by incubation on ice for 5 min.
3. Add 950 μ L of room temperature SOC outgrowth medium to the cell mixture and recover at $37^\circ C$, shaking at 250 rpm for 1 h.

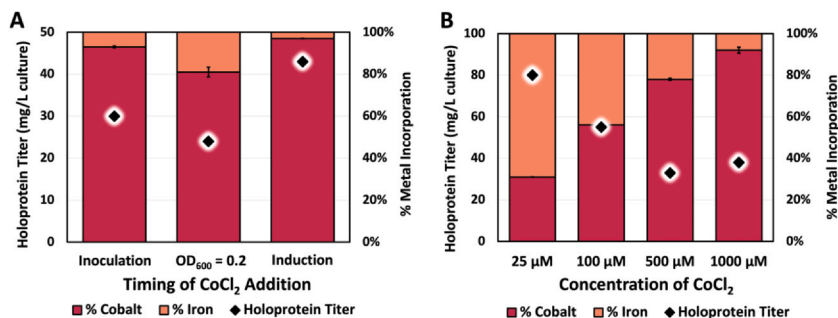


Fig. 2 Optimization of Co-incorporation and protein expression of dye decolorizing peroxidase (DyP) from *E. lignoliticus*. Protein expression was induced at an OD₆₀₀ ranging from 0.6 to 0.8. (A) Titters from DyP expression in BL21(DE3) with CoCl₂ added at inoculation, early log phase, or induction. Adding cobalt at the time of induction resulted in higher protein yield and improved metal incorporation. (B) Titters from DyP expression in BL21(DE3) cells with varying concentrations of CoCl₂. Higher cobalt concentrations led to decreased protein expression but significantly reduced iron contamination. Holoprotein titer was measured by pyridine hemochromagen assay, accounting for the ratio of Co versus Fe as determined by ICP-MS. Error represents triplicate ICP-MS measurements. % metal incorporation was measured by ICP-MS. Adapted with permission from Weaver, B. R., Perkins, L. J., Fernandez Candelaria, F. O., Burstyn, J. N., & Buller, A. R. (2023). ACS Synthetic Biology. Copyright 2023 American Chemical Society.

4. Transfer 50–100 μL of transformed cells to a 100 μg/mL ampicillin spiked LB agar plate.
5. Incubate inverted plates overnight at 37 °C.

3.3 Expression of cobalt-substituted hemoproteins

For maximum protein yield and Co-incorporation, we recommend adding 1 mM of CoCl₂ at the time of induction. Adding the toxic redox-active metal earlier results in significantly lower holoprotein titer as evident in Fig. 2A. Presumably, a higher cell density mitigates the stress of redox-active cobalt exposure by more rapidly reducing the concentration of extracellular cobalt in the media. Along these lines, inducing at a higher OD₆₀₀ also boosts protein titer, likely due to increased cell density at the time of cobalt supplementation and expression. While adding less than 1 mM CoCl₂ can increase holoprotein titer, it can increase iron contamination (Fig. 2B).

1. Inoculate 10 mL TB starter culture with a glycerol stock or colony of *E. coli* BL21(DE3) harboring the plasmid with the hemoprotein of interest.
2. Grow overnight at 37 °C, shaking at 200 rpm.

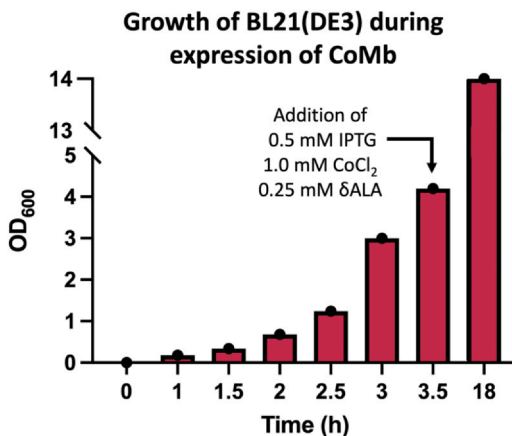


Fig. 3 Growth of BL21(DE3) expressing CoMb for a high-OD induction. Inoculated cell cultures were grown at 37 °C, shaking 220 rpm. The OD₆₀₀ was measured every 30 min after the first initial 1 h of growth. Once culture achieved an OD₆₀₀ ~ 5.0, approximately 4 h, cultures were placed on ice for 30 min. Cells were induced with 0.5 mM IPTG, 1.0 mM CoCl₂, and 0.25 mM δALA and proceeded to grow, shaking overnight at room temperature.

3. Add 10 mL (1 % inoculum) of the overnight starter culture to an auto-claved 2.8 L baffled flask, containing 1 L of TB and 100 µg/mL ampicillin.
4. Incubate the culture at 37 °C with shaking (220 rpm) until OD₆₀₀ reaches ~5. The doubling times for *E. coli* cells are typically around 30 min. Healthy cell cultures require at least 4 h of growing time. [Fig. 3](#) shows the growth rate of BL21(DE3) during expression of CoMb with a high-OD induction.
5. Once the OD₆₀₀ reaches ~5, cool the cell culture on ice for 30 min
6. Add 1000 µM CoCl₂ and 250 µM δALA to the culture, swirling by hand to fully incorporate.
7. Induce protein expression with 500 µM isopropyl β-D-1-thiogalactopyranoside (IPTG).
8. Incubate the culture at 20 °C overnight, shaking 180 rpm.
9. Harvest cells by centrifugation at 4000g for 30 min at 4 °C. Store the cell pellets in a -20 °C freezer until purification. For a typical 1 L expression, expect 20 g of wet cell pellet. The cell pellet should exhibit a distinct red color reflecting the presence of CoPPIX in the hemoprotein ([Fig. 5A](#)).

Note: M9 minimal medium is recommended for spectroscopic studies requiring low iron background. For preparative purposes or non-spectroscopic applications, rich media such as Hyper Broth or TB can be used

to maximize yield. Hyper Broth specifically has been observed to increase Co-substituted hemoprotein yields.

3.4 Purification of cobalt-substituted protein

1. Thaw cell pellet at room temperature.
2. Resuspend cells in lysis buffer containing 100 mM KPi (pH 7.0), 100 mM NaCl, 20 mM imidazole, 1 mg/mL lysozyme, 1 mM MgCl₂, 0.2 mg/mL DNase. Use a ratio of 4 mL lysis buffer per gram of wet cell pellet. With myoglobin, we recommend supplementing with 40 μ L CoPIX per g cell pellet, as any soluble apo-myoglobin that is non-metallated can bind this cofactor.
 - a. For less stable proteins, 10 % glycerol can be added to the lysis buffer to help solubility.
 - b. Protease inhibitor can be added to proteins that are prone to proteolysis.
3. Incubate for 30 min at 37 °C, shaking 200 rpm.
4. Sonicate the lysed cells for 30 s per g of cell pellet at 35 % amplitude using pulsing cycles of 1 s on and 3 s off.
 - a. For thermostable proteins such as CYP119, cells can be heat treated in place of sonication. Heat treat the cells in a 65 °C water bath for 45 min.
5. Clarify by centrifugation at 25,000g for 30 min at 4 °C.
6. Decant the clarified solution into a new tube or flask.
 - a. This clarified lysate can be used in reactions, instead of purified protein (Fig. 5B).
7. Load supernatant onto 2-5 mL of Ni-NTA column pre-equilibrated with storage buffer (100 mM KPi pH 7.0, 100 mM NaCl). Cycle the flow through over the column a second time to ensure complete binding of the His₆-tagged hemoprotein. The protein-loaded column should have a dark red color (Fig. 5C).
8. Wash with 2 column volumes of 20 mM imidazole in storage buffer and 2 column volumes of 40 mM imidazole in storage buffer.
 - a. Note: Washes with higher concentrations of imidazole can begin to prematurely elute the cobalt-substituted hemoprotein.
9. Elute the protein of interest from the column with storage buffer containing 250 mM imidazole with 3 column volumes or until the column is no longer red. The eluent should have distinct red color (Fig. 5D).

10. Imidazole can coordinate to the metal cofactor, inhibiting biocatalytic activity and interfering with the protein's spectroscopic signature. To ensure complete removal, dialyze the cobalt-substituted hemoprotein through five rounds of 1 L storage buffer, each for 2 h at 4 °C.
 - a. For less stable proteins, 10 % glycerol can be added to the dialysis buffer to prevent the protein from precipitating.
 - b. Alternatively, the eluted protein could be flown over a PD-10 desalting column pre-equilibrated in storage buffer, followed by fewer rounds of dialysis. The protein should be concentrated to less than 2.5 mL prior to loading the column.
11. Concentrate the protein using Amicon® Ultra 15 mL Centrifugal Filters (Millipore) in 5 min intervals of 4000 g at 4 °C.
 - a. Gently mix the protein between rounds to prevent aggregation and ensure a uniform sample.
 - b. Centrifugal filters with a molecular weight cutoff of 30 kDa was used for most hemoproteins, with the notable exception of myoglobin which had a molecular weight cutoff of 10 kDa.
12. Aliquot and flash freeze the concentrated protein in liquid nitrogen and store at −80 °C.
13. To analyze purity of the protein, run SDS-PAGE gel electrophoresis (Figs. 4 and 5).

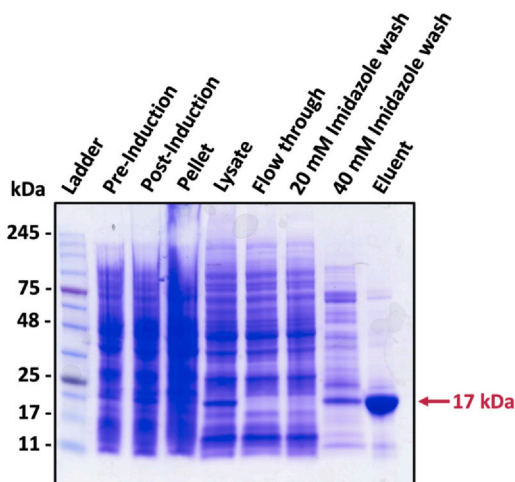


Fig. 4 SDS-PAGE analysis of CoMb (~17 kDa). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) is used to assess the purity of the cobalt-substituted enzymes. Eluent contains 250 mM imidazole.

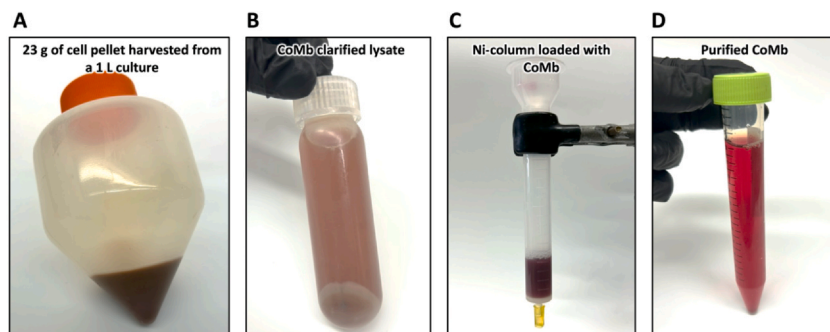


Fig. 5 Expression and purification of cobalt-substituted sperm whale Mb. (A) Cell pellet harvested from a 1 L culture expression of Mb supplemented with 1 mM CoCl_2 . (B) Clarified lysate containing CoMb. (C) 4 mL Ni-NTA column loaded with CoMb, indicated by the distinct red coloration of the resin. (D) Purified CoMb eluted from the Ni-NTA column.



4. Characterization of cobalt-substituted hemoproteins

4.1 Measuring concentration of cobalt-substituted hemoproteins

The concentration of Co-substituted hemoproteins can be readily measured by UV-vis spectroscopy using the pyridine hemochromagen assay (Berry & Trumpower, 1987). The reduced pyridine-CoPPIX complex exhibits a strong absorbance at 556 nm with an extinction coefficient of $9.7 \text{ mM}^{-1} \text{ cm}^{-1}$ (Fig. 6A).

1. Blank 700 μL of pyridine hemochrome reagent (40 mL pyridine, 36 mL H_2O , and 4 mL 1 M NaOH) in a quartz cuvette.
2. Add 50 μL of protein to the cuvette.
 - a. Volume of protein might need to be adjusted based on concentration.
3. Reduce CoPPIX with a few crystals of sodium dithionite, inverting to mix until reducing agent is fully incorporated.
4. Record UV-vis spectrum at 25 $^{\circ}\text{C}$ (Fig. 6B).
5. Calculate the concentration of CoPPIX using the absorbance of the reduced sample at 556 nm and the extinction coefficient of the pyridine-CoPPIX complex, $9.7 \text{ mM}^{-1} \text{ cm}^{-1}$.

4.2 Quantification of cobalt incorporation into hemoproteins

4.2.1 Qualitative assessment of porphyrin content with pyridine hemochromagen assay

The pyridine hemochromagen assay described above provides a useful qualitative assessment of metalloporphyrin content in purified protein

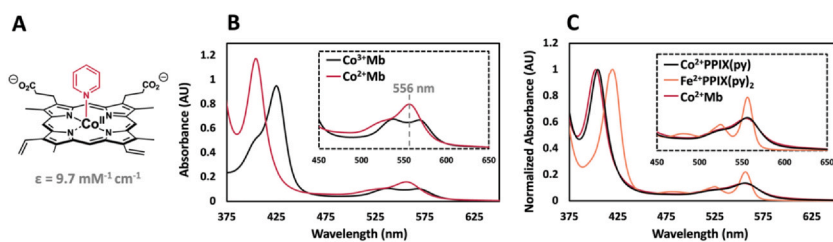


Fig. 6 Pyridine hemochromagen assay of CoMb to assess cobalt incorporation and protein concentration. (A) Structure of the pyridine–CoPPIX complex (CoPPIX(py)) complex corresponding to the red trace in the electronic absorption spectrum. The molar absorptivity at 556 nm is $9.7 \text{ mM}^{-1} \text{ cm}^{-1}$ which is used to calculate the protein concentration. (B) Electronic absorption spectra from the pyridine hemochrome assay of Co-substituted myoglobin. The black trace shows the spectrum of CoMb in the hemochrome reagent, and the red trace shows the spectrum after reduction with sodium dithionite. The concentration of this CoMb sample was $589 \text{ }\mu\text{M}$, corresponding to a protein yield of 50 mg/L . (C) Comparison of the normalized electronic absorption spectra of $\text{Co}^{2+}\text{PPIX}$ (black trace), $\text{Fe}^{2+}\text{PPIX}(\text{py})_2$ (orange trace), and the CoMb derived sample (red trace). The spectrum of the CoMb derived sample clearly aligns with the CoPPIX(py) signal.

samples (Majtan et al., 2011). For more precise analysis, the samples can be heated to 70°C to fully denature the protein and form the pyridine metalloporphyrin complex. As seen in Fig. 6C, $\text{Co}^{2+}\text{PPIX}(\text{py})$ and $\text{Fe}^{2+}\text{PPIX}(\text{py})_2$ exhibit unique spectral features in the Soret and Q-band region of the UV–vis spectrum, allowing for quick identification of the presence of CoPPIX compared to FePPIX in purified protein samples.

4.2.2 UPLC-MS analysis of porphyrin content in purified protein

Another method to quantify the porphyrin speciation of a hemoprotein is by UPLC-MS analysis. Briefly, the protein is denatured to release the porphyrins, and a sample containing the porphyrin is injected onto the UPLC-MS. Porphyrins are detected by absorbance at their Soret maxima (398 nm for FePPIX, 400 nm for H_2PPIX , 423 nm for CoPPIX) and by positive single ion reads (SIR): $616.3 \text{ } m/z$ (FePPIX), $563.3 \text{ } m/z$ (H_2PPIX), and $619.3 \text{ } m/z$ (CoPPIX), as shown in Fig. 7. Porphyrin content can be quantified using the integrated UV absorbance and comparison to a standard curve. To calculate the concentration of porphyrin in the original sample, multiply the concentration of porphyrin in the analyte solution by the volume of the organic supernatant.

1. To prepare the purified protein sample for UPLC-MS, mix $2\text{--}30 \text{ }\mu\text{L}$ of purified protein with $200 \text{ }\mu\text{L}$ of an 8:2 pyridine to 1.2 M HCl solution.
2. Vortex the mixture for 1 min.

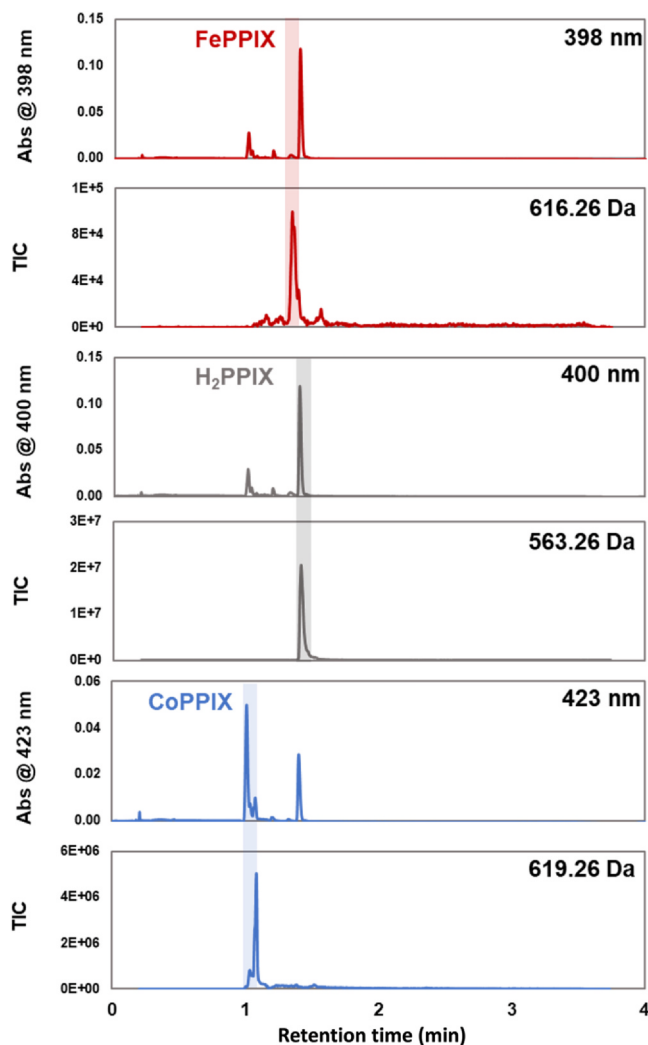


Fig. 7 Example of a UPLC-MS trace for a purified protein sample. The trace shown is for a single replicate of a purified sample of CoPPIX-substituted dye-decolorizing peroxidase (CoDyP), which has a particularly high H₂PPIX loading. Total ion counts (TIC) at the mass of the $m+1$ ions align well with the Soret absorbance maxima for each porphyrin. Note that H₂PPIX and FePPIX have similar Soret absorbance maxima. Mass detection confirms peak identity of the FePPIX at a slightly shorter retention time than H₂PPIX. Retention times will vary depending on chromatographic methodology. Reprinted with permission from Perkins, L. J., Weaver, B. R., Buller, A. R., & Burstyn, J. N. (2021). PNAS.

3. Add 200 μL of saturated MgSO_4 to the solution and vigorously vortex again.
4. Centrifuge the emulsion at 4300 g for 5 min at 4 $^{\circ}\text{C}$ to separate aqueous and organic layer.
5. Remove the organic layer and clarify once again by centrifugating at 14,000 rpm for 3 min.
6. Inject 3 μL of the organic soluble portion onto the UPLC-MS. The UPLC chromatographic method should consist of an isocratic mobile phase (25 % water in acetonitrile with 0.1 % formic acid), flowing at a rate of 1 mL/min over an ACQUITY UPLC-CSH Phenyl-Hexyl column, 130 $\text{\AA}$ 1.7 μm , 2.1 $\times$ 50 mm (Waters Part no. 186005406).

4.2.3 ICP-MS analysis for total metal content of purified proteins

Inductively coupled plasma-mass spectrometry (ICP-MS) offers an alternative method to quantify the metal content of protein samples by comparing measured values to a standard curve. Table 1 shows the calculated porphyrin, metal, and holoprotein concentrations for a variety of purified hemoproteins samples.

1. To prepare the purified protein sample for ICP-MS, add 350 μL of trace analysis grade 70 % nitric acid to < 30 μL of purified protein.
2. Incubate the sample at 80 $^{\circ}\text{C}$ for 4 h to fully digest.
3. Dilute the sample to a final concentration of 5 % nitric acid with ultrapure water.
4. Prepare cobalt and iron standard solutions in 5 % nitric acid from 1000 ppm atomic absorption standards.

4.3 Spectroscopic analysis of cobalt-substituted hemoproteins

Cobalt-substituted hemoproteins display distinct spectroscopic features compared to their Fe-containing counterparts. These unique characteristics provide a window into studying hemoprotein function. For instance, electron paramagnetic resonance (EPR) analysis of cobalt-substituted sperm whale myoglobin (CoMb) provided valuable insights into the structure and properties of the paramagnetic CoMb- O_2 complex (Chien & Dickinson, 1972; Hoffman & Petering, 1970; Yonetani et al., 1974). Similarly, resonance Raman spectroscopy was used to detect the O-O stretching mode in oxygen-bound CoMb and cobalt-substituted hemoglobin, made possible by the enhancement of this vibrational mode of the cobalt-substituted proteins compared to the native Fe-proteins (Bruha & Kincaid, 1988; Mackin et al., 1983; Proniewicz & Kincaid, 1990). The unique spectral

Table 1 Calculated porphyrin, metal, and holoprotein concentrations for purified protein samples. Comparison of relative porphyrin content and % holoprotein for hemoproteins expressed in cobalt-supplemented media and rich media. Note that DyP contains significant quantities of free base protoporphyrin IX (H2PPIX) when expressed in either Fe- or Co-containing media. These proteins samples were obtained from expressions in M9* media, with hemoprotein expression induced at an OD₆₀₀ ranging 0.6 to 0.8.

Protein	Relative porphyrin content			Relative metal content			Yield (mg protein/L culture) ^b
	CoPIX	FePIX	H ₂ PPIX	Co	Fe	% Holoprotein ^a	
CoMb*	>99 %	0.1 ± 0.1 %	0.1 ± 0.1 %	99.8 ± 0.3 %	0.2 ± 0.3 %	29 ± 2 %	4.7
CoCYP119	99 ± 1 %	<1 %	<1 %	>99.4 %	<0.6 %	101 ± 2 %	3.6
CoOxd	>99 %	0.3 ± 0.7 %	<3 %	95.9 ± 0.9 %	4.1 ± 0.9 %	63 ± 1 %	3.7
CoCat	99 ± 1 %	0.8 ± 0.1 %	<0.1 %	>99 %	<1 %	54 ± 1 %	2.2
CoDyP	74 ± 1 %	1.1 ± 0.4 %	25 ± 1 %	99.4 ± 0.4 %	0.6 ± 0.4 %	49 ± 0.2 %	6.5
FeDyP	<5 %	70 ± 2 %	25 ± 1 %	NA ^c	NA	22 ± 3 ^d	NA

^aPercentage of [Co] (measured by ICP-MS) versus [protein] (measured by BCA assay)
^bCalculated as mg of holoprotein per L culture. Yields were reproducible within a factor of 2 between batches.
^cNA, not assessed
^dPercentage of [heme] (measured by pyridine hemochrome assay) versus [protein] (measured by BCA assay)
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features resulting from cobalt-substitution also enable the characterization of novel reactivity modes that arise from cobalt incorporation (Epping et al., 2022; L. Perkins et al., 2024).

The porphyrin cofactor of hemoproteins exhibits rich spectral signatures in the electronic absorption spectrum (Nienhaus & Nienhaus, 2005). The two main electronic absorption features of a metalloporphyrin are the intense Soret peak in the near-visible region and the smaller Q-bands in the visible region, both resulting from $\pi \rightarrow \pi^*$ transitions (Gouterman et al., 1963; Gouterman, 1959, 1961). The precise wavelength and shape of these peaks depend on the nature of the metalloporphyrin, including the metal identity, oxidation state, and axial ligands. For example, sperm whale Mb features a native histidine as its axial ligand (Fig. 8A and B) and displays distinct spectral characteristics when comparing its native Fe-containing form to the Co-substituted variant. The λ_{max} for the Soret peak of the Fe^{3+} -containing enzyme appears at 413 nm while the Q-bands are observed at 507, 538, 584, and 627 nm (Fig. 8C). Upon reduction with a few crystals of sodium dithionite and inverting to mix, the Soret peak redshifts to 432 nm, and the Q-band region features a prominent peak at 556 nm. Cobalt-substitution significantly alters the spectral features of the metalloporphyrin. In CoMb, the Soret peak appears at 427 nm and the Q-bands are at 536 and 576 nm (Fig. 8E). The reduced Co^{2+} Mb spectrum exhibits a blueshifted Soret peak at 407 nm and a Q-band peak centered at 556 nm. These spectra highlight the sensitivity of the metalloporphyrin to its coordination environment, metal identity, and oxidation state, making electronic absorption spectroscopy a powerful tool for investigating both native and new-to-nature intermediates and reactivities of hemoproteins.

Cobalt(II)-substituted hemoproteins display informative electron paramagnetic resonances (EPR) spectra. Unlike ^{56}Fe , which has a nuclear spin of 0, ^{59}Co has a nuclear spin of 7/2, resulting in hyperfine coupling interactions in the EPR spectrum. This hyperfine coupling reveals the distribution of unpaired electron spin density on the Co^{2+} metal center, providing insight into the covalency of the Co^{2+} -ligand bonds. Due to the low spin ($S=1/2$) $3d^7$ electron configuration of Co^{2+} in the cobalt-substituted hemoproteins, featuring an unpaired electron in the d_{z^2} orbital, EPR spectroscopy serves as an exceptionally sensitive tool for probing axial ligand interactions. In contrast, the EPR spectrum of the native Fe-containing myoglobin provides rather limited information, merely indicating that the high-spin ($S=5/2$) Fe^{3+} complex is reduced to a low-spin ($S=0$) Fe(II) diamagnetic species (Fig. 8D). The cobalt-substituted analog offers complementary insights into the ligand environment characterized by the Spin Hamiltonian parameters obtained from spectral fits, including g - and

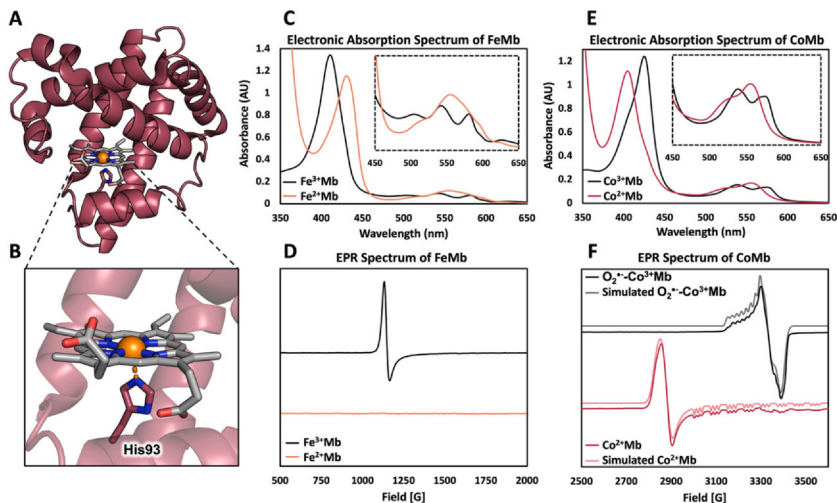


Fig. 8 Spectroscopic analysis of cobalt-substituted Mb compared to native FeMb. (A) Crystal structure of sperm whale Mb (PDB: 1VXA). (B) Active site of Mb showing the His93 axial ligand coordinated to the metalloporphyrin. (C) and (D) Electronic absorption and EPR spectra, respectively, of 5 μM as-isolated (black trace) and reduced (orange trace) native Fe-containing Mb in 100 mM KPi (pH 7.0) and 100 mM NaCl. EPR spectra were obtained using 0.02 mW (Fe³⁺Mb) and 2.0 mW (Fe²⁺Mb) microwave power, 100 kHz modulation frequency, 5 G modulation amplitude, 60 dB receiver gain, 40.96 s time constant, 40.96 s conversion time at 20 K. (E) Electronic absorption spectra of 20 μM as-isolated (black trace) and reduced (red trace) Co-substituted Mb in 100 mM KPi (pH 7.0) and 100 mM NaCl. (F) EPR spectra of 20 μM as-isolated (black trace) and reduced (red trace) Co-substituted Mb in 100 mM KPi (pH 7.0) and 100 mM NaCl. The simulated spectrum for the Co³⁺-superoxide species is shown in gray and that for Co²⁺Mb is shown in light red. EPR spectra were obtained using 0.6325 mW (Co³⁺Mb) and 2.0 mW (Co²⁺Mb) microwave power, 100 kHz modulation frequency, 5 G modulation amplitude, 60 dB receiver gain, 40.96 s time constant, 40.96 s conversion time at 20 K. The EPR spectrum of as-isolated CoMb reveals a characteristic Co³⁺-superoxide species, indicating oxygen coordination to the metal. The reduced CoMb EPR spectrum shows hyperfine coupling of Co²⁺ with the nitrogen of the axial His ligand.

A-values. To illustrate this point, we show in Fig. 8F how the EPR spectrum of Co²⁺Mb exposed to O₂ displays eight hyperfine lines, consistent with a low-spin, 6-coordinate Co³⁺-superoxide species (Hoffman & Petering, 1970; Ikeda-Saito et al., 1978). This spectrum demonstrates that oxygen binds to the Co²⁺ as the upper axial ligand thus mimicking the native mechanism of myoglobin. The EPR spectrum of reduced Co²⁺substituted Mb (Fig. 8F) also shows eight hyperfine features that are further split into triplets due to coupling to the axially coordinated ¹⁴N nucleus (nuclear spin 1). This pattern confirms that the cobalt is coordinated by a nitrogen donor ligand, likely His93. Spin Hamiltonian parameters derived from simulations of these spectra are provided in Table 2. In the

Table 2 Spin Hamiltonian parameters derived from simulations of the EPR spectra of CoPIX-substituted myoglobin species.

Protein	Spin state	Ligand	g-values			Cobalt hyperfine coupling (MHz)			Ligand hyperfine coupling (MHz)		
			g_x	g_y	g_z	A^{Co_x}	A^{Co_y}	A^{Co_z}	$A^{N_{XY}}$	A^{N_z}	
$O_2^{\bullet-}$ -Co ³⁺ Mb	1/2	His/O ₂	2.08	2.02	1.98	60	N/R ¹	N/R	N/A ²	N/A	
Co ²⁺ Mb	1/2	His	2.32	2.32	2.04	N/R	N/R	213	N/R	48	

¹Not resolved
²Not available

Co^{3+} -superoxide species, the unpaired spin density primarily resides on the nascent superoxide ligand, whereas in Co^{2+}Mb , the unpaired electron is localized on the cobalt metal center, as evidenced by the cobalt hyperfine coupling constants. Overall, cobalt-substituted hemoproteins provide valuable structural and electronic insights through their EPR spectra, enhancing our understanding of metal-ligand interactions and the coordination environment. EPR spectroscopy of these systems serves as a powerful tool for investigating the structure of intermediates involved in both native enzymatic mechanisms and engineered biocatalytic pathways.



5. Conclusions

Cobalt-substituted hemoproteins are valuable tools for mechanistic enzymology and the development of novel biocatalysts. Replacing the native iron center with cobalt allows researchers to manipulate the redox properties, ligand binding affinities, and electronic structures of heme-containing enzymes. Cobalt-substitution is particularly important in the context of biocatalysis, where metal substitution can unlock reactivities that are inaccessible with the native iron. For example, cobalt can stabilize different oxidation states or facilitate alternative reaction pathways. These cobalt-substituted hemoproteins exhibit distinct spectroscopic features, which enables tracking of metal coordination environments with UV-vis and EPR spectroscopy. Previous methods to acquire cobalt-substituted hemoproteins have been hampered by labor-intensive cofactor synthesis, variable metal incorporation, and low yields. Our approach leverages the *in vivo* biosynthesis of CoPPIX and its incorporation into hemoproteins using *E. coli* BL21(DE3) cells. These easy-to-implement methods are readily adapted to produce either small quantities of highly pure cobalt-substituted hemoproteins from minimal media for sensitive spectroscopic applications, or > 95 % Co-loaded hemoproteins in appreciable yields from rich media—sufficient for biocatalytic applications.

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Declaration of AI and AI-assisted technologies in the writing process

During the preparation of this work the authors used ChatGPT in order to increase the readability of the text. After using this tool/service, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

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