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Supporting Information

for

Ferritin Encapsulation of Artificial Metalloenzymes: Engineering a Tertiary Coordination Sphere for an Artificial Transfer Hydrogenase

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Experimental

General

All commercially available chemicals were purchased from commercial suppliers (Acros Organics, Alfa Aesar, AnaSpec, AppliChem, Fluka, Merck, NEB, Sigma-Aldrich) and used without further purification. Solvents for HPLC measurements were purchased from Baker and Biosolve. Horse spleen apoferritin (HsAf) was purchased from Sigma Aldrich and subjected to gel filtration prior to use. Biotin-4-fluorescein (B4F) was purchased from Sigma-Aldrich and used directly without purification.

Size exclusion chromatography was performed on an Äktaprime Plus chromatography system, using a HiLoad 16/60 Superdex 200 PG column from GE Healthcare (preparative runs) or a Wyatt 4.6x300mm, 300 Å Pore, Silica SEC Column (analytical runs).

Dynamic light scattering (DLS) was performed using a Malvern Instruments Zetasizer Nano ZS Dynamic Light Scattering Instrument.

Mili-Q water (resistivity ≥ 18 MΩ cm^{-1}) was produced with a Millipore Synergy purification system. Streptavidin (Sav) mutants were produced, purified and characterized as previously described. [1]

Analyses of the catalytic runs were performed on an Agilent 1100 normal phase HPLC with an analytical Chiralpack IC column (250 · 4.6 mm, 5 µm). ICP-MS analysis was performed on a 7500cx ICP-MS system (Agilent, Basel, Switzerland).

Stock solutions and buffers

2x MOPS/formate buffer: 3-(*N*-morpholino)propanesulfonic acid and sodium formate were dissolved in Milli-Q water to a concentration of 6M (formate) and 1.2 M (MOPS). The pH was adjusted to 8 by addition of NaOH.

Tris-HCl buffer: Tris base and NaCl were dissolved in Milli-Q water to a concentration of 50 mM (Tris) and 100 mM (NaCl). The pH of the solution was adjusted to 8 by the addition of 37% HCl.

*Catalyst **3** stock solution:* [Cp*Ir(biot-*p*-L)Cl)] was dissolved in DMF to a final concentration of 500 mM. For a detailed synthesis procedure, see reference. [2]

Substrate stock solutions:

- 6,7-dimethoxy-1-methyl-3,4-dihydroisoquinoline **1a** was dissolved in Mili-Q water (2 ml) to a final concentration of 1 M.
- 1-phenyl-3,4-dihydroisoquinoline **1c** (synthesis procedure see reference [3]) was dissolved in degassed DMSO (2 ml) to a final concentration of 400 mM.

Sav preincubation and lyophilisation

In a typical experiment, purified Sav mutant (25 mg , approx. 3,8 x 10^{-7} mol) was dissolved in Milli-Q water. The average number of free binding sites per purified Sav tetramer was determined with a biotin-4-fluorescein assay, as described elsewhere. [4] The corresponding amount of [Cp*Ir(biot-*p*-L)Cl] ligand stock solution was added to produce protein with binding sites half filled with the iridium cofactor (ratio of Ir : free binding sites 1 : 2). Samples were incubated (37 °C, 2 h), frozen (-80°C) and lyophilised to dryness.

Apoferritin encapsulation

The apoferritin sample purified my gel filtration was subjected to the re-assembly process. The protein solution was diluted to a concentration of 30 mg / mL and the pH of the solution was decreased below 2 using 37% HCl. The resulting solution was incubated for 20 minutes at 600 rpm at RT followed by the addition of a lyophilized ATHase. The pH of the resulting solution was increased to 8 using 10 M NaOH solution. After incubation for 2 hours at RT, iminobiotin-sepharose beads were added and the solution was incubated for further 1 h at rapid shaking (800 rpm). After binding the non-encapsulated Sav to beads, the solution was centrifuged (5300 g, 15 min), the supernatant was filtered and subjected to another 2 rounds of immobilization. The resulting cargo-containing ferritin samples were further purified by a small-scale dialysis (Spectra/Por, 6-8.000 MWCO, against 50 mM Tris-HCl, 100 mM NaCl, pH 8.0) to remove the free cofactor and by size-exclusion chromatography.

Size-exclusion chromatography

In order to confirm the loading of the guest, Sav S112A-K121A was incubated with biotin-4-fluorescein and encapsulated in HsAf following the above-mentioned encapsulation route. The purified samples were subjected to SEC (HiLoad 16/60 Superdex 200 PG; 50 mM Tris-HCl, 100 mM NaCl, pH 8.0, 25°C, 0.4 mL / min) and monitored at λ=280 and 495 nm.

ATHases bearing mutations S112A, S112K and S112A-K121A were encapsulated and purified following the same procedure. The fractions corresponding to the ATHase@ferritin signal (elution volume around 68 mL, Figure S1) were pooled, concentrated using a Amicon Ultra -15 centrifugal filter unit with a 30 kDa cut-off (Merck Millipore) and stored at 4°C prior to analysis by ICP-MS and catalysis.

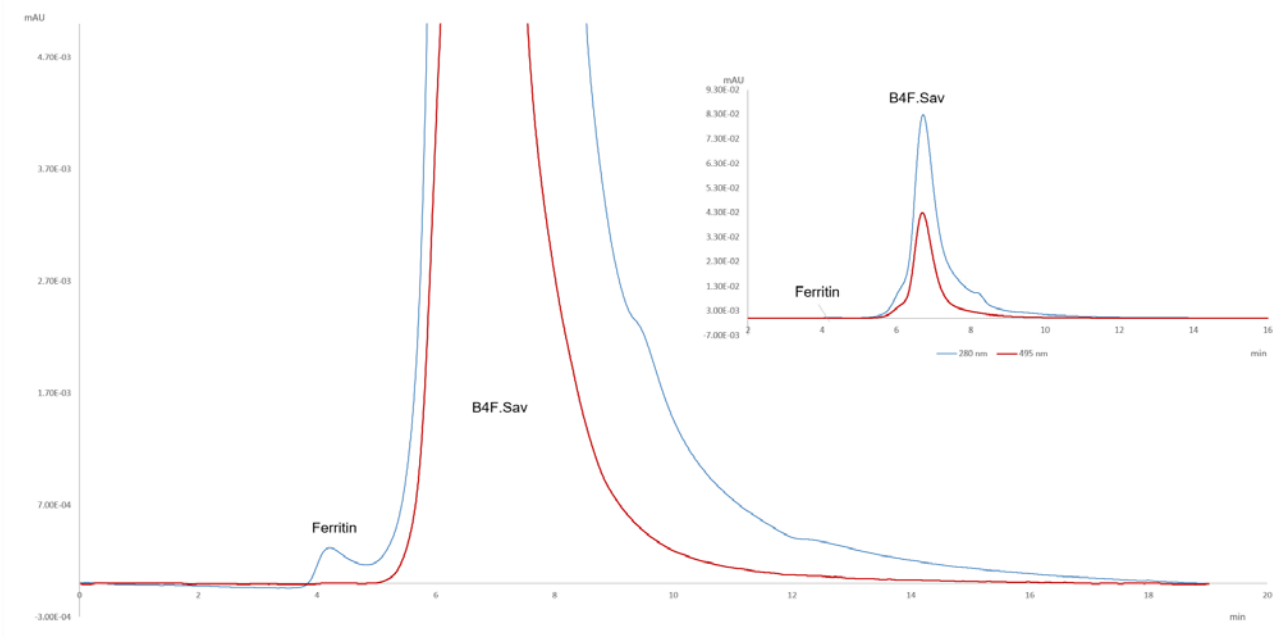


Figure S1. Analytical size-exclusion chromatogram of a mixture of B4F@ferritin and B4F·S112A-K121A. The blue line indicates absorbance at 280 nm. Run in 50 mM Tris-HCl, 100 mM NaCl, pH 8.0). Column: Wyatt 4.6x300mm, 300 Å Pore, Silica SEC Column.

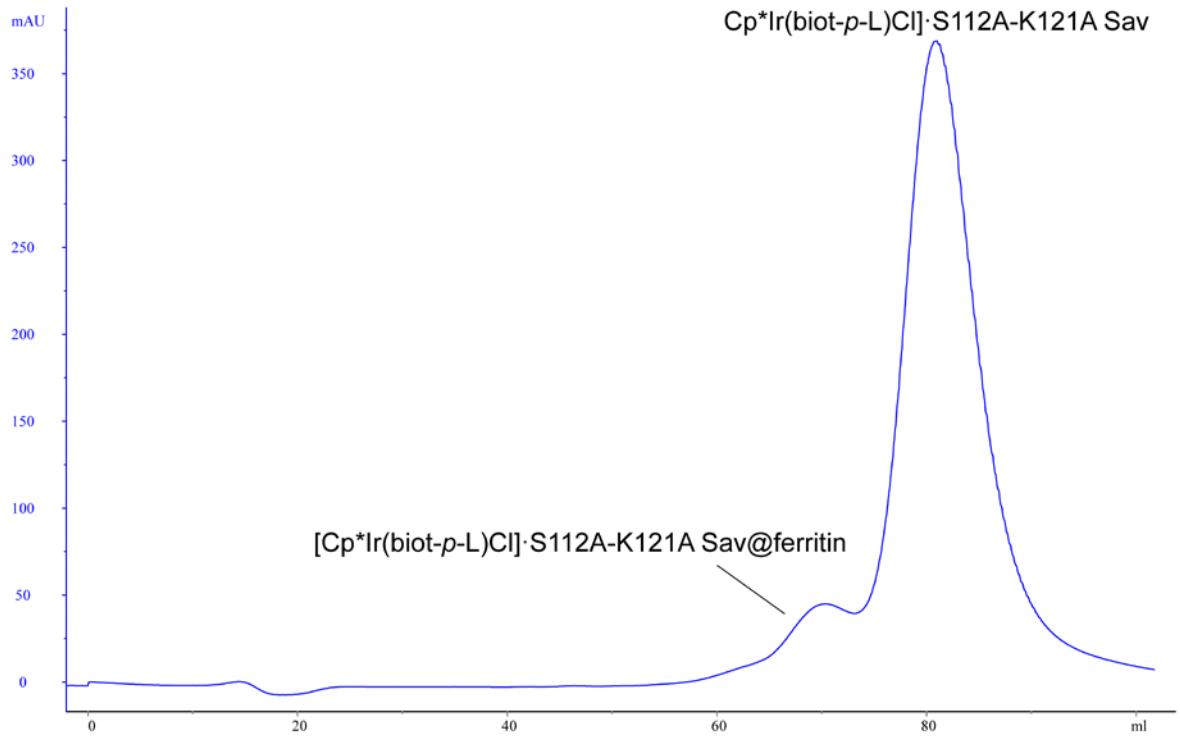


Figure S2. Preparative size-exclusion chromatogram of [Cp*Ir(biot-*p*-L)Cl]·S112A-K121A Sav@ferritin. The blue line indicates absorbance at 280 nm. Run in 50 mM Tris-HCl, 100 mM NaCl, pH 8.0). Column: HiLoad 16/60 Superdex 200 PG.

Dynamic light scattering measurements (DLS)

Dynamic light scattering (DLS) measurements of the combined fractions containing the assembled ferritin were performed on a Malvern Instruments Zetasizer Nano ZS Dynamic Light Scattering Instrument. Sample analysis was performed with purified ferritin samples at an approx. concentration of 0.1 mg/ml in 50 mM Tris-HCl, 100 mM NaCl, pH = 8 using a low-volume quartz cuvette. All solutions were filtered through a 0.2 µm filter to prevent further aggregation.

Table S1. Average size of apoferritin and ferritin samples containing ATHase cargo

entry	Sample	Diameter	PDI
1	Apoferritin	13.43	0.052
2	[Cp*Ir(biot-*p*-L)Cl]·S112A-K121A Sav@ferritin	15.71	0.488
3	[Cp*Ir(biot-*p*-L)Cl]·S112A Sav@ferritin	17.66	0.658
4	[Cp*Ir(biot-*p*-L)Cl]·S112K Sav@ferritin	14.44	0.783

PDI = polydispersity index

Electrophoresis

SDS-PAGE

The pooled samples from SEC purification were concentrated using a Amicon Ultra -15 centrifugal filter unit with a 30 kDa cut-off (Merck Millipore) and the protein concentration was determined by Nanodrop2000 (ThermoFisher) using $\varepsilon_{280\ nm}$= 4.8x10^5 M^{-1}cm^{-1}). The gel analysis was performed according to a published procedure [7] using hand-casted 10% acrylamide gels, followed by B4F detection under UV and staining with Coomassie Brilliant Blue. The stacking layer (6% final acrylamide concentration) was prepared at pH = 6.8.

20 µl of each sample type (diluted so the final amount of loaded protein would be 2 µg per lane) was combined with a 6x loading buffer and either heated to 90°C for 10 min prior to the addition of 1 µl of 0.6 M B4F stock solution in DMSO and loaded. Alternatively, the samples were prepared without the heating step.The gel was ran at 200 V until for approx. 1.5 hours. After B4F visualization the gels were stained with Coomassie Brilliant blue R-250 dye in MeOH and acetic acid, followed by de-staining.[5]

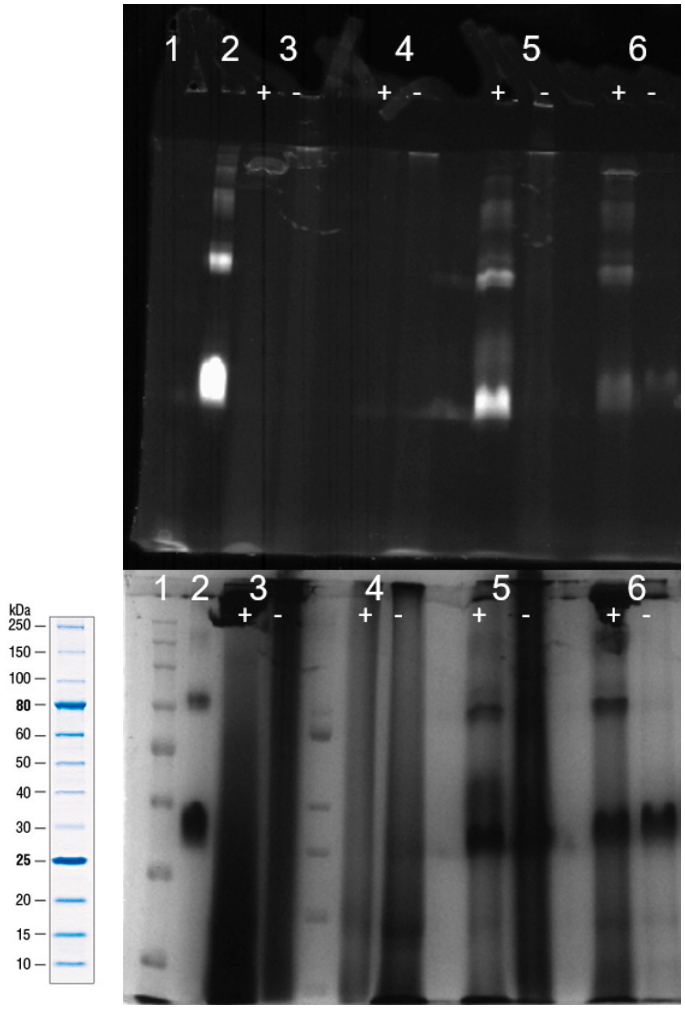


Figure S3. SDS-PAGE gel under UV (B4F visualization) and Vis light (Coomassie staining). + indicates heating during sample preparation, - indicates semi-native conditions (i.e. absence of heating) Lane 1: ladder; 2: Sav WT (control); 3: apoferritin; 4: reassembled apoferritin; 5: [Cp*Ir(biot-*p*-L)Cl]·S112A-K121A Sav@ferritin; 6: [Cp*Ir(biot-*p*-L)Cl]·S112A

Sav@ferritin. Remark: As Sav is not fully denatured and retains its globular form under these conditions, it migrates at around 35 kDa.

Native PAGE

The pooled samples from SEC purification were concentrated using a Amicon Ultra -15 centrifugal filter unit with a 30 kDa cut-off (Merck Millipore) and the protein concentration was determined by Nanodrop2000 (ThermoFisher) using $\varepsilon_{280\ nm}$= 4.8x10^5 M^{-1}cm^{-1}). The gel analysis was performed according to a published procedure [7] using hand-casted 10% native acrylamide gels, followed by B4F detection under UV and staining with Coomassie Briliant Blue. The stacking layer (6% final acrylamide concentration, no SDS added) was prepared at pH = 6.8.

20 µl of each sample type (diluted so the final amount of loaded protein would be 2 µg per lane) was combined with a 6x loading buffer (without DTT or mercaptoethanol) and 1 µl of 0.6 M B4F stock solution in DMSO and directly loaded. The gel was ran at 200 V until for approx. 1.5 hours. After B4F visualization, the gels were stained with Coomassie Brilliant Blue R-250 dye in MeOH and acetic acid, followed by de-staining.

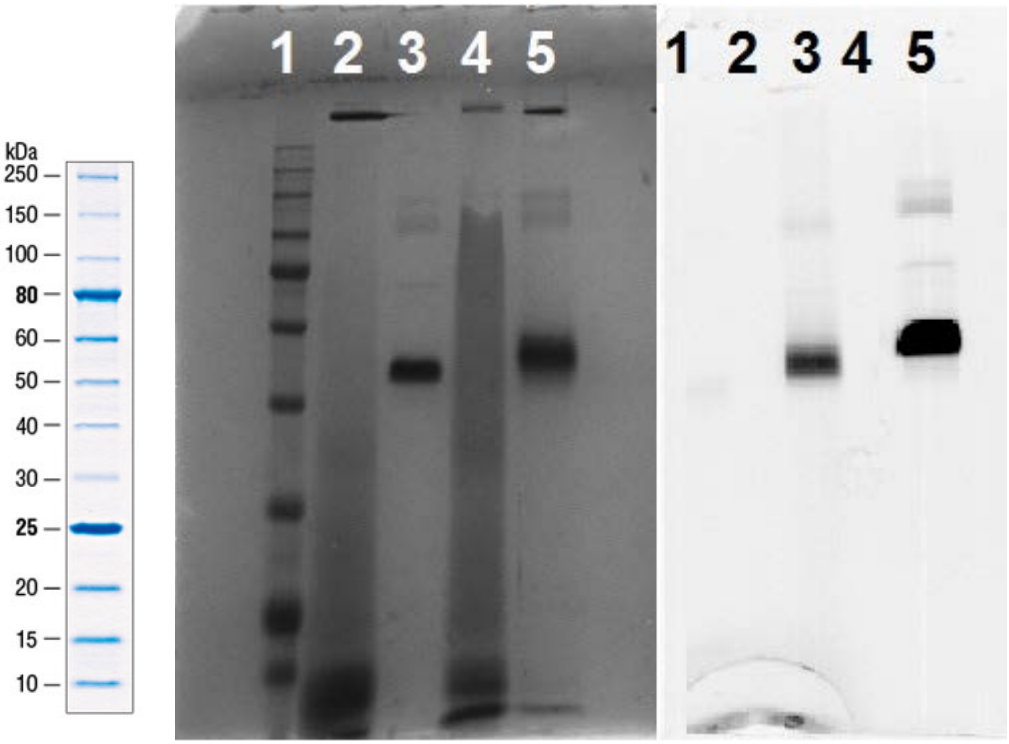
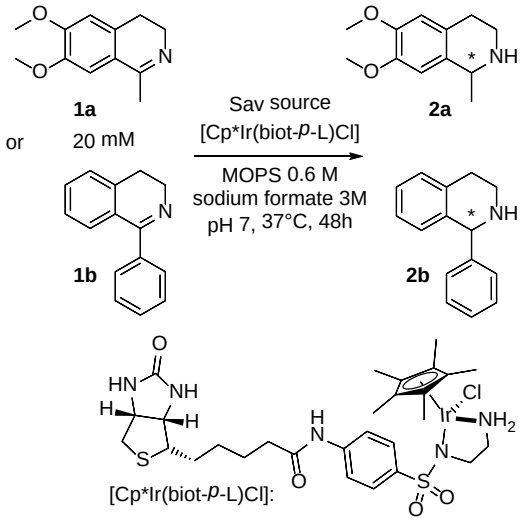


Figure S4. Native-PAGE gel under Vis (Coomassie staining) and UV (B4F visualization). Lane 1: ladder; 2: apoferritin sample; 3: Sav S112A-K121A; 4: [Cp*Ir(biot-*p*-L)Cl]·S112A-K121A Sav@ferritin; 5: apoferritin + Sav S112A-K121A. Remark: Sav migrates at around 65 kDa.

ICP-MS determination of the total Ir content

Fractions from SEC containing the ferritin species were combined and the sample (10 µl) was incubated with concentrated HNO_3 solution (65 %, semiconductor grade, Sigma Aldrich) at 95°C for 30 min. The resulting samples were diluted to an final concentration of 3 % HNO_3 using Milli-Q water. Analysis was performed on a 7500cx ICP-MS system (Agilent, Switzerland) using standard operational settings as described elsewhere[6]. Quantification was perfomed via multi-element standards (Sigma-Aldrich) in matrix-matched calibration solutions containing the same amount of HNO_3 as the samples. Rh was used as internal standard to account for residual matrix effects. ^{191}Ir was used for quantification, ^{193}Ir for verification of the results. The octopole was pressurized (4.5 mL min^{-1} helium) to reduce polyatomic interferences.

Catalysis



Scheme S1. Catalysis set up

In a typical catalysis procedure, the cofactor, an ATHase or ATHase@ferritin solution (45 µl) were diluted with 2x MOPS / formate buffer (50 µl, final concentration 0.6 M MOPS, 3 M sodium formate, pH 8.0) in a HPLC vial. The reaction was initiated by addition of a substrate stock solution (5 µl, end concentration 20 mM). The vials were sealed and the reaction mixtures were shaken at 600 rpm in a Thermoshaker at 37°C (final volume 100 µl). Each reaction mixture was diluted with Milli-Q water (400 µl) and basified using NaOH solution (20 %, 50 µl). The resulting mixture was extracted with dichloromethane (2 x, 1 ml), the combined organic fractions were collected in a PP tube containing anhydrous sodium sulfate, and centrifuged (18800 x g, 5 min). The supernatant was analyzed by means of HPLC.

HPLC measurements

Catalysis results for substrate **1a** were analyzed using a Chiralpak IC column (5 µm, 4.6 mm · 25 mm) and dichloromethane containing diethylamine (0.06 %) and isopropanol (0.5 %) as an eluent (1 ml / min flow; λ = 280 nm, 25 °C).

T_R 8.5 min ((*S*)- 6,7-dimethoxy-1-methyl-1,2,3,4-tetrahydroisoquinoline), 9.8 min (6,7-dimethoxy-1-methyl-3,4-dihydroisoquinoline), 14.6 min ((*R*)- 6,7-dimethoxy-1-methyl-1,2,3,4-tetrahydroisoquinoline). Yields were calculated using a response factor of 1.95, as determined elsewhere.[8]

Screening with substrate **1c** was analyzed using a Chiralpak IC column (5 µm, 4.6 mm · 25 mm) and hexane containing diethylamine (0.06 %) and isopropanol (3 %) as an eluent (1 ml / min flow; λ=265 nm, 25 °C). T_R 7.6 min ((*S*)-1-phenyl-1,2,3,4-tetrahydroisoquinoline, 10.5 min ((*R*)-1-phenyl-1,2,3,4-tetrahydroisoquinoline), and 16 min (1-phenyl-3,4-dihydroisoquinoline). Yields were calculated using a response factor of 13.613 as determined elsewhere.[8]

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