

Supporting Information

Synthesis of hybrid transition-metalloproteins via thiol-selective covalent anchoring of Rh-phosphine and Ru-phenanthroline complexes

René den Heeten, Bianca K. Muñoz, Gina Popa, Wouter Laan* and Paul C. J. Kamer*

Site-directed mutagenesis and cloning

ALBP C1A

The C1A mutant of ALBP was constructed with the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). pRSET-ALBP¹ was used as the template for PCR, and the primer sequences were:

5' CCTCGAAGGTTTACAAAATGGCCGATGCCTTTGTGGGAACC 3' and

5' GGTCCCACAAAGGCATCGGCCATTTTGTAACCTTCGAGG 3'

The construct (pRSET-ALBP C1A) was verified by sequencing (The Sequencing Service, Dundee, UK).

To clone the ALBP gene into pQE30, cDNA corresponding to the ALBP C1A mutant was amplified by PCR using pRSET-ALBP C1A as template, using the primers:

5'-GCACGGATCCGATGACGATGACAAAATGGCCGATGCCTTTGTGG-3'

(BamHI site underlined, enterokinase site in italics) and

5'-GCACCTGCAGTCATGCCCTTTCATAAACTCTTG-3'

(PstI site underlined)

The PCR product was purified from agarose gel (Wizard SV Gel and PCR Clean-Up System, Promega), digested with BamHI (Fermentas) and PstI (Fermentas) and ligated into BamHI/Ps I digested pQE30 vector (Qiagen) using T4 DNA ligase (Fermentas). The construct was verified by sequencing (The Sequencing Service, Dundee, UK).

Protein expression and purification

PYP and PYP R52G

These proteins were expressed and purified as previously described.²

ALBP C1A

E. coli M15 pREP4 transformed with pQE-ALBP C1A was grown at 37°C in PB containing ampicillin and kanamycin. When the OD₆₀₀ reached ≈ 3, protein expression was induced by adding IPTG to a final concentration of 0.5 mM and growth was continued for 16 hours at 20°C. The cells were then harvested by centrifugation (4°C, 20 min, 3000 g) and resuspended in ice-cold lysis buffer (50 mM phosphate buffer, 50 mM NaCl, 10% glycerol, 0.5% Nonidet P-40,²¹ pH 8.0) containing protease inhibitor (Complete EDTA free, Roche). The cell-suspension was treated with deoxyribonuclease (30 μg ml⁻¹), ribonuclease (30 μg ml⁻¹), and lysozyme (1 mg ml⁻¹), held on ice for 30 min, sonicated (10 minutes with a 50% duty cycle) and centrifuged (4°C, 30 min, 27000 g). The soluble fraction was applied to a 5ml HisTrap™ FF column (GE Healthcare Life Sciences), the column was washed first with washing buffer (50 mM phosphate, 150 mM NaCl, 20 mM imidazole, 10% glycerol, pH 8.0) containing 0.5% detergent (Nonidet P-40)²¹, and then with an equivalent volume of washing buffer without detergent. The protein was eluted with elution buffer (50 mM phosphate, 150 mM NaCl, 500 mM imidazole, pH 8.0) and dialyzed overnight at 4°C against 50 mM phosphate, 50 mM NaCl, pH 8.0 and stored at -20°C. Protein concentration was determined using the Bradford assay². Purity of the samples was checked by SDS-PAGE and was routinely more than 95%.

Whole protein mass spectrometry using ESI-TOF

The protein sample (20 µl, 10-20 pmol/µl) was desalted on-line through a XTerra MS C8 2.1 x 10 mm column, eluted with an increasing acetonitrile concentration (2% acetonitrile, 98% aqueous 1% formic acid to 98% acetonitrile 2% aqueous 1% formic acid) and delivered to an electrospray ionisation mass spectrometer (LCT, Micromass, Manchester, U.K.) which had previously been calibrated using myoglobin. The electrospray source of the LCT was operated with a capillary voltage of 3.5 kV and a cone voltage of 25 V. An envelope of multiply charged signals was obtained and deconvoluted using MaxEnt1 software to give the molecular mass of the protein species present. For PYP and PYP R52G, frequently peaks corresponding to apo-protein with an extra mass of 16 and/or 32 Da were observed; these are assigned to singly or doubly oxidized apo-protein, likely due to oxidation of cysteine and/or methionine during the analysis:

Calculated masses apo-proteins:

PYP WT : apo = 15861.8, singly oxidized = 15877.8, doubly oxidized = 15893.8

PYP R52G: apo = 15762.4, singly oxidized = 15778.4, doubly oxidized = 15794.4

ALBP: apo = 16604.8

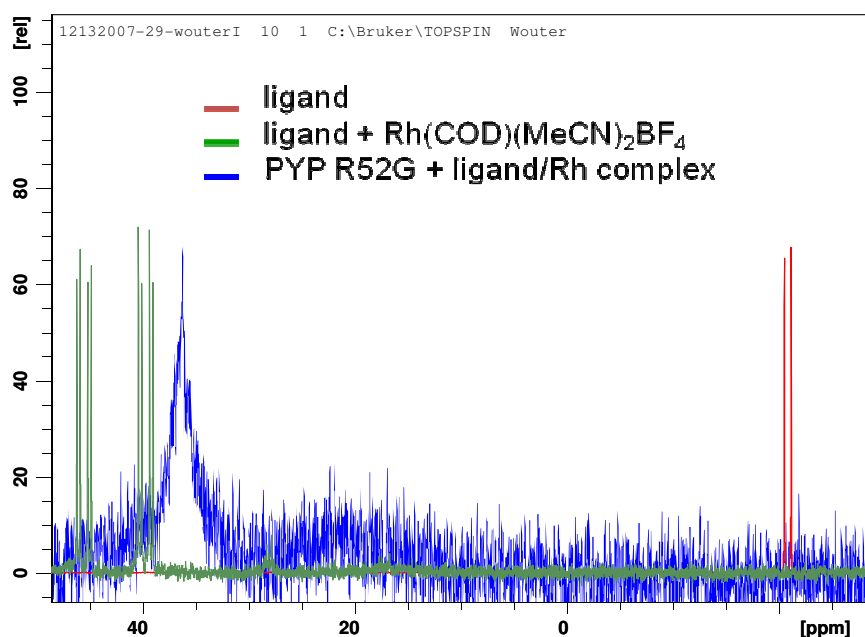


Figure S1 ^{31}P NMR of rhodium complexes bearing ligand **4**. a) free ligand in red, b) $[(4)\text{Rh}(\text{cod})]\text{BF}_4$ in green and c) PYP R52G- $[(4)\text{Rh}(\text{cod})]\text{BF}_4$ in blue.

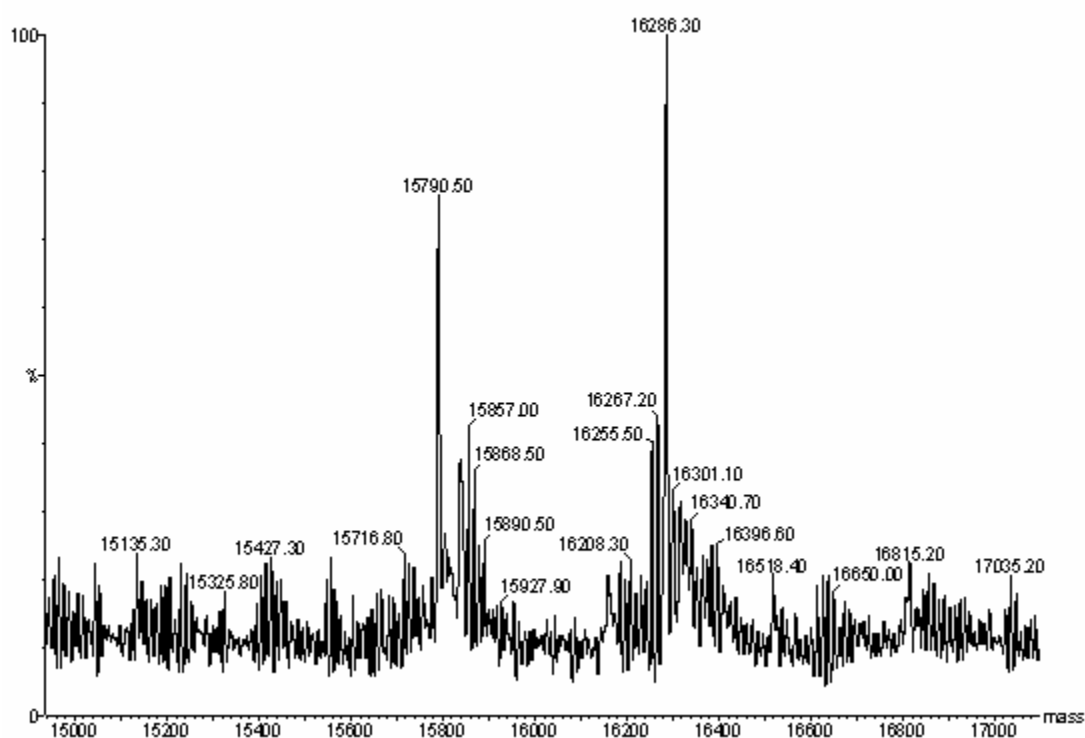


Figure S2 LC-MS spectrum of PYP R52G modified with $[\text{Ru}(\text{OH}_2)(\text{phen-maleimide})(p\text{-cymene})](\text{OTf})_2$. The observed masses correspond to the mass of oxidized protein (15790.5 Da) and the protein modified with $[\text{Ru}(\text{OH}_2)(\text{phen-maleimide})(p\text{-cymene})]$ (16286.3 Da)

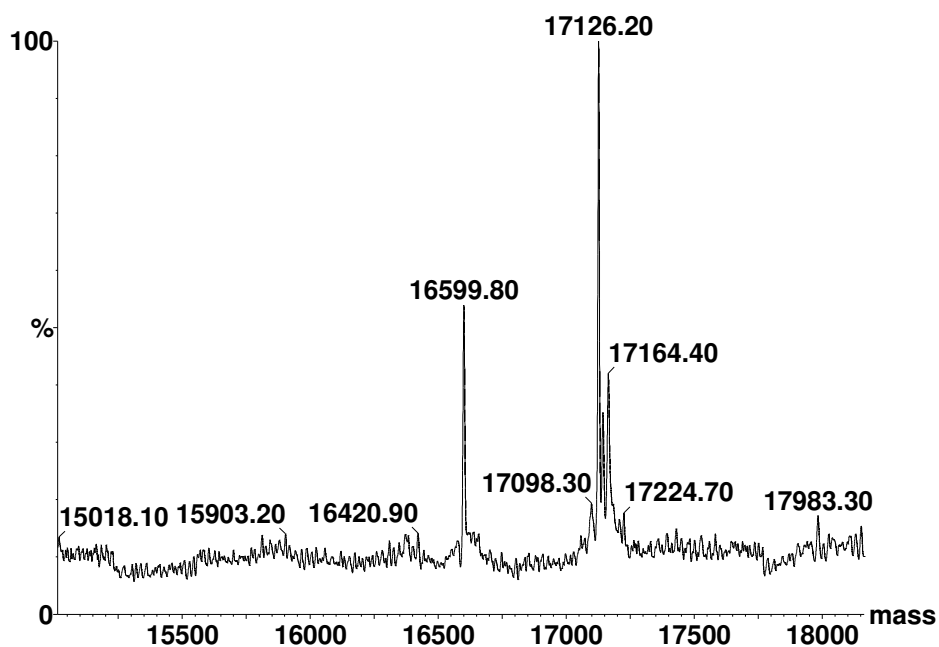


Figure S3 LC-MS spectrum of ALBP modified with $[\text{Ru}(\text{OH}_2)(\text{phen-maleimide})(p\text{-cymene})](\text{OTf})_2$. The observed masses correspond to the mass of apo-protein (16599.8 Da) and the protein modified with $[\text{Ru}(\text{OH}_2)(\text{phen-maleimide})(p\text{-cymene})]$ (17126.2 Da)

Tryptic Digests

The samples (5 μL , 10 pmoles/ μL) were dialysed against buffer (50 mM ammonium bicarbonate pH 8.0) using a membrane filter (Millipore, Billerica, MA). Trypsin (0.5 μL , 0.1 μg , Promega, Madison, WI) was added to the resulting solutions. The solutions were incubated at 37°C overnight and the digested solution (0.5 μL). This solution together with an alpha-cyano-4-hydroxycinnamic acid matrix (0.5 μL , 10 mg/mL in 50:50 acetonitrile:0.1% TFA) and 0.1% TFA (0.5 μL) was applied to the MALDI target, and after being allowed to dry, analyzed by MALDI-MS. MALDI MS was acquired using a 4800 MALDI TOF/TOF Analyser (Applied Biosystems, Foster City, CA) equipped with a Nd:YAG 355 nm laser and calibrated using a mixture of peptides. The samples were initially analysed in positive MS mode between 800 and 4000 m/z, by averaging 1000 laser shots. The most desired peptides were selected for MS/MS analysis and acquired to a maximum of 3000 laser shots or until the accumulated spectrum reached a S/N ratio of 35. All MS/MS data were acquired using 1 keV collision energy.

The combined MS and MS/MS data were analysed, using GPS Explorer (Applied Biosystems) to interface with the Mascot 2.1 search engine (Matrix Science, London, UK), against the UniProt (Swiss-Prot and TrEMBL combined) database (April 2009). No species restriction was applied. The data were searched with tolerances of 100 ppm for the precursor ions and 0.5 Da for the fragment ions, with trypsin as the cleavage enzyme, assuming up to one missed cleavage, carbamidomethyl modification of cysteines as a fixed modification and methionine oxidation selected as a variable modification. Digest results are shown for PYP WT-9 in figure S4 and for ALBP-9 in figure S6. The depicted peptides were the only peptide found matching modification with the complex.

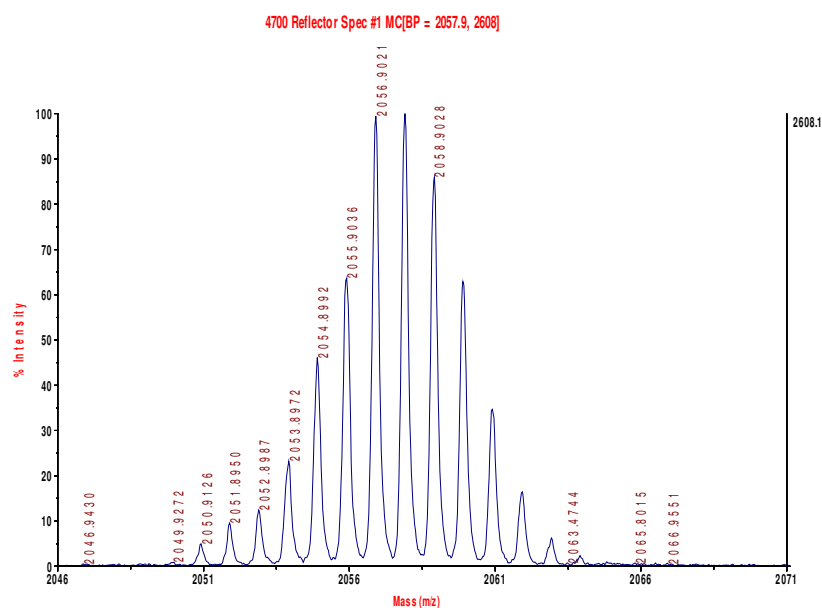


Figure S4 Experimental LC-MS spectrum of fragment KDVAPCTDSPEFYG of PYP WT modified with [Ru(OH₂)(phen-maleimide)(*p*-cymene)] obtained by trypsin digestion of PYP WT-9. The observed signal at 2056.9 Da corresponds to the molecular weight of the peptide (1528.6 Da) modified with 9 (528.5 Da)

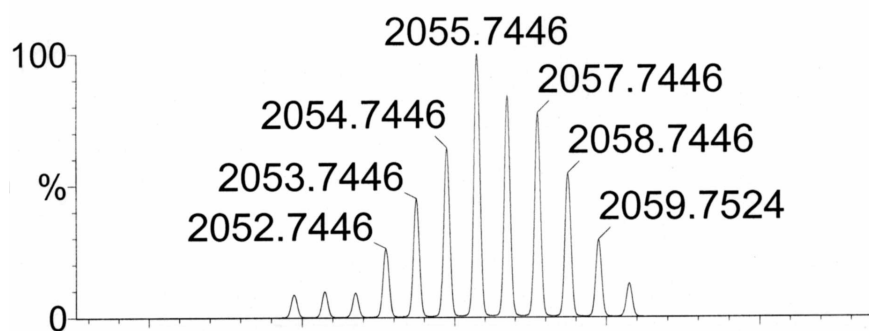


Figure S5 Calculated LC-MS spectrum of fragment KDVAPCTDSPEFYG of PYP modified with [Ru(OH₂)(phen-maleimide)(*p*-cymene)]

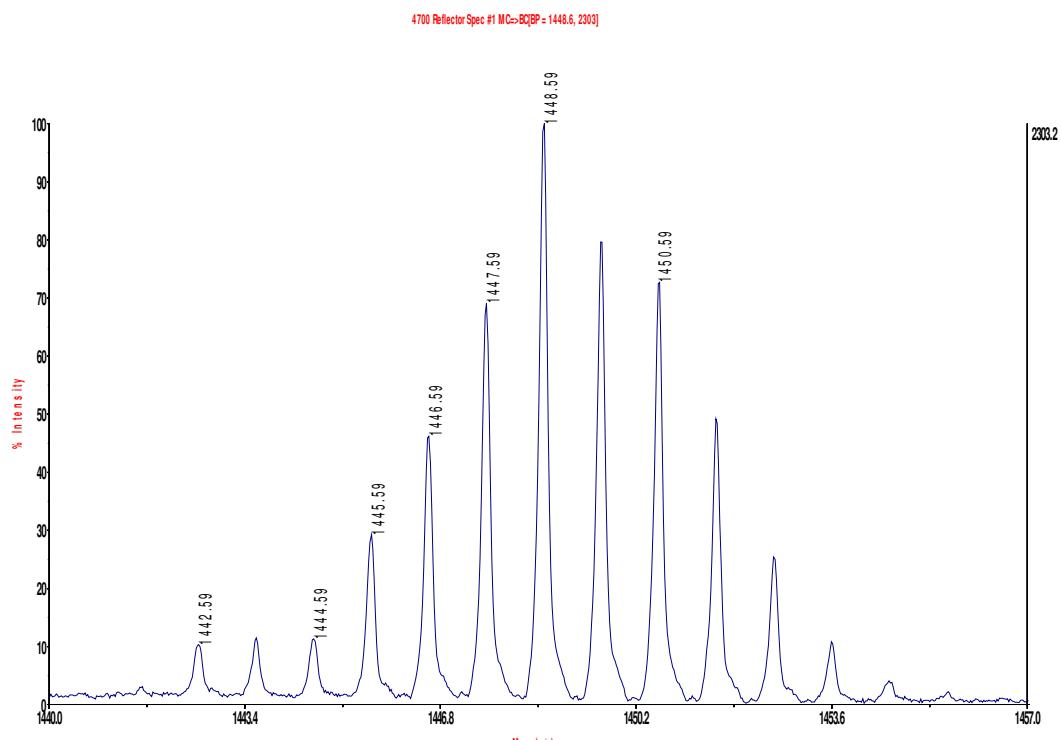


Figure S6 Experimental LC-MS spectrum of fragment KLVVECVM of ALBP modified with [Ru(OH₂)(phen-maleimide)(*p*-cymene)] obtained by trypsin digestion of ALBP-9. The observed signal at 1448.6 Da corresponds to the molecular weight of the peptide (920.1 Da) modified with **9** (528.5 Da)

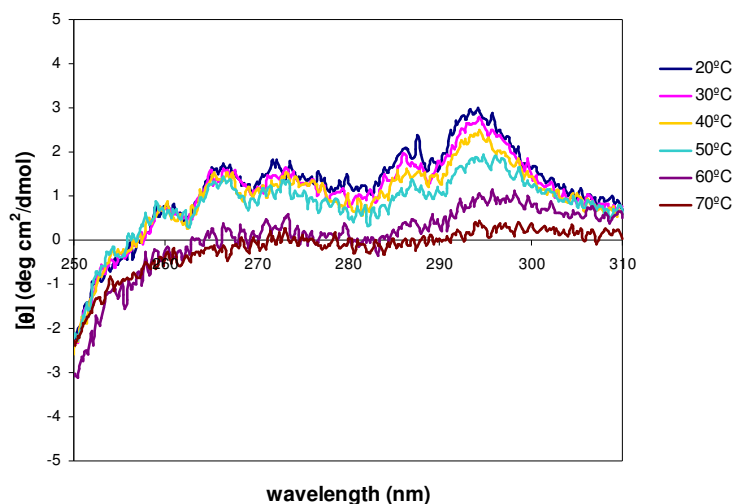


Figure S7 Circular dichroism spectra of PYPWT modified with $[\text{Ru}(\text{OH}_2)(\text{phen-maleimide})(p\text{-cymene})](\text{OTf})_2$

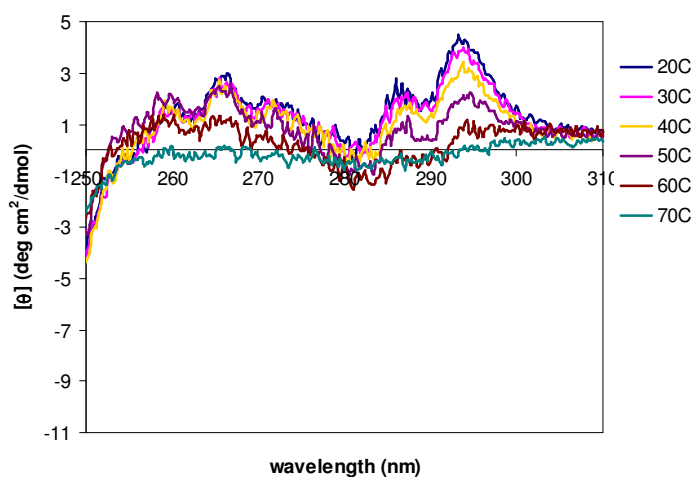


Figure S8 Circular dichroism spectra of PYPR52G modified with $[\text{Ru}(\text{OH}_2)(\text{phen-maleimide})(p\text{-cymene})](\text{OTf})_2$

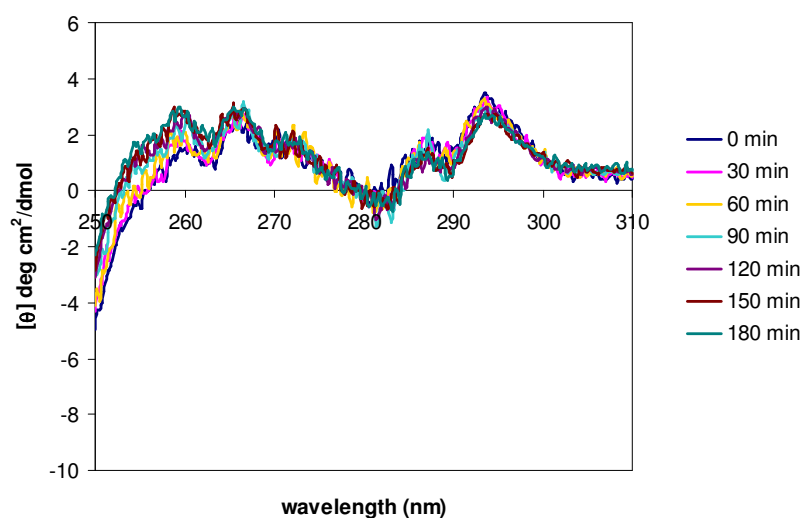
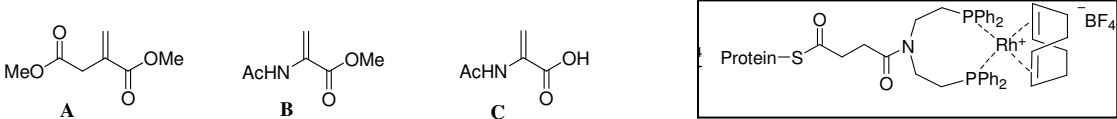


Figure S9 Circular dichroism spectra of PYPR52G modified with $[\text{Ru}(\text{OH}_2)(\text{phen-maleimide})(p\text{-cymene})](\text{OTf})_2$ at 40°C

Hydrogenation of alkenes

The hydrogenation reactions were carried out in a stainless steel autoclave with glass inserts at a hydrogen pressure of 5 bar. The results and conditions are summarized in Table S1. In the hydrogenation of dimethyl itaconate **A** using the R52G-rhodium catalyst no conversion was observed. The addition of organic cosolvents such as DMF and DMSO did have a positive effect on the activity of the artificial metalloenzyme. When the hydrogenation reaction of itaconate **A** was performed in a mixture of DMF and buffered aqueous solution (1:1) full conversion was observed, but enantioselectivity was low. We assume that the protein partially denatures upon addition of organic solvents resulting in a more accessible transition metal complex. In the hydrogenation of acrylate **B** and acrylic acid **C** no conversions were obtained, not even when organic cosolvents were used.

Table S1. Results of asymmetric hydrogenation.^[a]



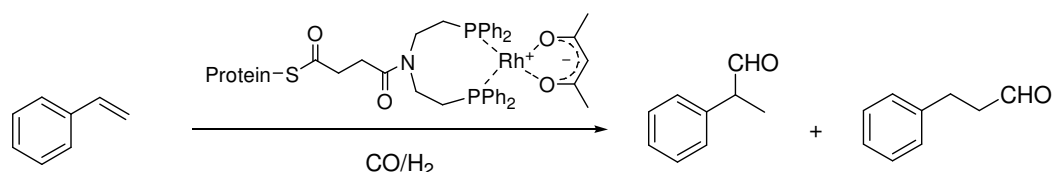
Entry	Substrate	Solvent ^[b]	Conv. (%) ^[c]	ee (%) ^[d]
1	A	-	0	-
2	A	DMF	> 99	6
3	A	DMSO	> 99	3
4	B	DMSO	0	-
5	C	DMSO	0	-

[a] Conditions: [Rh] = 56 μ M, substrate/Rh = 100, $p(\text{H}_2)$ = 5 bar, T = 25 °C, t = 20 h, total volume: 0.3 mL. [b] Reactions were performed in MOPS buffer (100 mM, pH 7.0) or HEPES buffer (100 mM, pH 7.0) or where noted in mixtures (1:1) with organic solvents. [c] Percentage conversion of substrate, determined by GC. [d] Enantiomeric excess of product, determined by chiral GC.

General procedure for asymmetric hydrogenation experiments. The hydrogenation experiments were carried out in a stainless steel autoclave (total volume is 150 mL) charged with an insert suitable for 8 or 14 glass reaction vessels with Teflon mini stirring bars for conducting parallel reactions. In a typical hydrogenation run, a glass vial was charged with degassed buffer solution containing the artificial metalloenzyme complex (56 μ M) and substrate. Before starting the catalytic reactions, the charged autoclave was purged three times with 5 bar of dihydrogen and then pressurized to 5 bar H_2 . The reaction mixtures were stirred at 25 °C for 20 h. Next, the autoclave was depressurized and each reaction mixture was extracted with EtOAc (3 \times 5 mL) and the combined organic layers were dried over Na_2SO_4 and concentrated under reduced pressure. The residue was redissolved in ethyl acetate (100 μ L) and transferred into a micro-GC vial. The conversion was determined by GC measurement and the enantiomeric excess was measured by chiral GC using the following columns and conditions: for **A**: Supelco β -DEX 225 column (T = 70 °C for 50 min, then $\Delta T = 25$ °C min^{-1} , t_R (S) = 51.7 min, t_R (R) = 52.3 min, t_R (A) = 53.5 min); and for **B**: Chiralsil DEX-CB column (T = 70 °C for 1 min, then $\Delta T = 7$ °C min^{-1} , t_R (B) = 6.4 min, t_R (S) = 7.2 min, t_R (R) = 7.4 min). For the reaction with acrylic acid **C**, the reaction mixture was after the appropriate reaction time quenched by adjusting the pH to 2 with 1 M aqueous HCl. The aqueous solution extracted with ethyl acetate (6 \times 1 mL) and the combined organic layers were concentrated under reduced pressure to a minimum volume (0.5 mL). The hydrogenation product was converted *in situ* to its methyl ester using trimethylsulfonium hydroxyde (15 μ L) before GC analysis.

Hydroformylation of styrene

The PYP R52G modified with **4**-Rh(acac) was evaluated in the biphasic hydroformylation of styrene. Biphasic hydroformylation reactions are generally performed at temperatures of 40-80 °C and under pressures of 50-80 bar of syngas. A very small number of chiral water-soluble ligands has been used in the Rh-catalyzed asymmetric hydroformylation of styrene. In all cases, the enantioselectivities obtained for the branched product were rather low (up to 25 % ee).



Styrene and pentane as cosolvent were added to a concentrated protein solution and the hydroformylation reaction was performed at 20 bar of syngas (CO/H₂ = 1) at 40 °C. Under these relative mild conditions no conversion was observed. Increasing the pressure to 40 bar of syngas did not have a beneficial effect. When the reaction was performed under 40 bar of syngas and at 80 °C still no conversion was observed, however the reaction mixture had turned into a white, cloudy mixture after 20 hours. The high temperature and pressure of syngas applied might cause denaturation of the protein that can result in the formation of the observed aggregates.

General procedure for the hydroformylation of styrene. The hydroformylation experiments were carried out in a stainless steel autoclave (total volume is 150 mL) charged with an insert suitable for 8 or 14 glass reaction vessels with Teflon mini stirring bars for conducting parallel reactions. Styrene was filtered over basic alumina to remove possible peroxide impurities. In a typical experiment, a reaction vessel was charged with the R52G-rhodium complex (6.7 mM in 0.50 μmol) and water (180 μL). A mixture of styrene (75 μmol) and decane as internal standard (37.5 μmol) in pentane (75 μL) was added. The autoclave was flushed three times with 15.0 bar of syngas (CO/H₂ = 1), warmed to 40 °C and then pressurized to 20.0 bar CO/H₂. After the appropriate reaction time, the magnetical stirrer was stopped, the reactor cooled rapidly and the autoclave was depressurized. The organic layer was separated. The conversion was determined by GC using a DB-1 (J&W) column (70 °C for 1 min, then ΔT₁ = 7 °C min⁻¹ to 120 °C and ΔT₂ = 13 °C min⁻¹ to 250 °C; retention times: 9.0 min for styrene, 11.3 min for decane, 12.9 min for 2-phenylpropanal and 13.8 min for 3-phenylpropanal. The enantiomeric purity was determined by chiral GC using a Supelco β-DEX 225 column (T = 100 °C for 5 min, then ΔT = 4 °C min⁻¹, t_R (branched *R*) = 11.8 min, t_R (branched *S*) = 12.1 min, t_R (linear) = 15.6 min).

Transfer hydrogenation of acetophenone

The transfer hydrogenation reactions were carried out at pH 6.5 and at 40°C. A series of control reactions were performed to verify that no conversion can be obtained using isolated reaction components. Although a low conversion was expected under these mild conditions, traces of product were detected when the ruthenoproteins were used as catalysts. The main drawback associated with this reaction is the harsh conditions required to get reasonable conversions.

General procedure for the asymmetric transfer hydrogenation. The following procedure is a modification from the procedure previously reported by T. Ward and coworkers.³ GC sample vials, containing a stirring bar, were placed in a carousel and degassed. 550 µL of the formate mixture (stock solution [HCOONa] = 1 M + [H₃BO₃] = 0.85 M), 340 µL of MilliQ Water and 10 µL of the catalyst (stock solution 13 mM in water) were mixed and stirred during 10 min. 100 µL of substrate (stock solution [acetophenone] = 130 mM in DMSO) was then added to the previous solution and the reaction mixture was heated with stirring at 40 °C for 70 hours. The reaction was stopped by cooling it down to 0 °C with an ice/water bath. The aqueous phase was extracted with diethyl ether (4 x 1 mL, extraction was performed by adding the Et₂O to the vial, vortexing the vial for 30 sec, followed by centrifugation for 1 min at 3000 g in a 15 ml Falcon centrifugation tube (BD Biosciences) to facilitate phase-separation, dried with sodium sulphate and filtered through a short silica gel plug. The solvent was removed using a rotavap and the residue was redissolved in 400 µL of a hexane/iso-propanol mixture (1:1) to analyse by chiral HPLC (Chiralcel-OD-H; n-hexane/2-propanol = 95:5 at 0.5 ml min⁻¹, λ 254 nm, acetophenone 12.5 min, (+)-phenylethanol 17.5 min, (-)-phenylethanol 21.7 min.

Preparation of the boric acid/sodium formate solution: Boric acid (1.05 g, 17 mmol) and sodium formate (1.36 g, 20 mmol) were dissolved in water (20 mL). The pH was adjusted to 6.5 with NaOH pellets and the solution was thoroughly degassed. The final stock-solution concentration was H₃BO₃ = 0.85 M, HCOONa = 1 M.

References

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3. (a) C. Letondor, N. Humbert and T. R. Ward, *Proc. Natl. Acad. Sci. USA*, 2005, **102**, 4683; (b) C. Letondor, A. Pordea, N. Humbert, A. Ivanova, S. Mazurek, M. Novic and T. R. Ward, *J. Am. Chem. Soc.*, 2006, **128**, 8320