This article is published as part of the *Dalton Transactions* themed issue entitled:

Bridging the gap in catalysis *via* multidisciplinary approaches

Guest Editors: Christophe Coperet and Rutger van Santen
Université de Lyon, France and Eindhoven University of Technology, The Netherlands

Published in issue 36, 2010 of *Dalton Transactions*

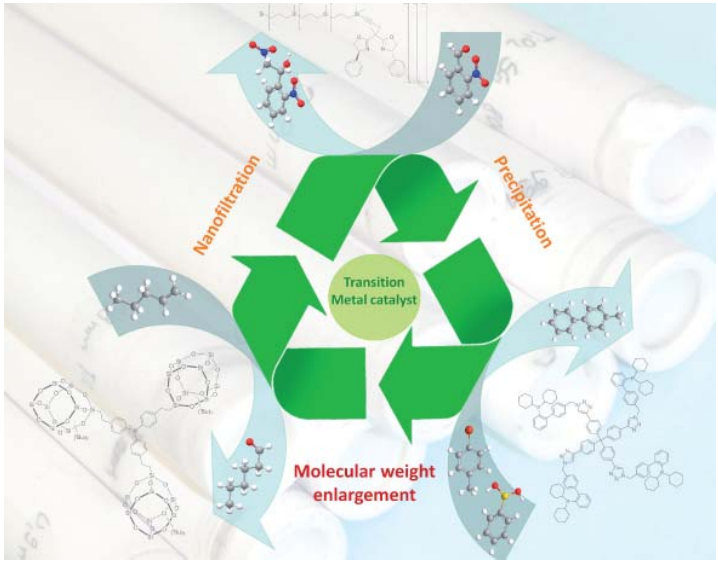


Image reproduced with the permission of Dieter Vogt

Articles in the issue include:

Visit the *Dalton Transactions* website for more cutting-edge inorganic and organometallic research
www.rsc.org/dalton

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

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

Received 31st March 2010, Accepted 10th June 2010
DOI: 10.1039/c0dt00239a

The preparation of hybrid transition metalloproteins by thiol-selective incorporation of organometallic rhodium- and ruthenium complexes is described. Phosphine ligands and two rhodium-diphosphine complexes bearing a carboxylic acid group were coupled to the cysteine of PYP R52G, yielding a metalloenzyme active in the rhodium catalyzed hydrogenation of dimethyl itaconate. The successful coupling was shown by ^{31}P NMR spectroscopy and ESI mass spectroscopy. In addition wild-type PYP (PYP WT), PYP R52G and ALBP were successfully modified with a (η^6-arene) ruthenium(II) phenanthroline complex *via* a maleimide linker.

Introduction

Enantioselective catalysis is one of the most efficient strategies for the preparation of enantiopure compounds. Catalytic reactions can be achieved by either chemocatalysis or biocatalysis. In the field of chemocatalysis, homogeneous catalysis with transition metals has received significant attention during the past decades and has proven useful and versatile in numerous enantioselective transformations.[1] Both the nature of the metal center and the coordinating ligand determine the properties of the catalyst.[2] The key to the success of organometallic catalysts lies in the relative ease of catalyst modification by changing the ligand environment. In biocatalysis, the use of enzymes for organic transformations has received increasing attention.[3] The excellent chemo-, regio- and enantioselectivity and activity displayed by enzymes, in combination with the introduction of directed evolution methodologies for performance optimization[4] lie at the basis of this interest. Furthermore, enzymes offer an environment-friendly alternative to toxic chemical reagents and the field of green chemistry is, at present, enjoying a high level of attention from the scientific community.

With the aim to combine the best of both homogeneous catalysis and biocatalysis, the development of artificial metalloenzymes has received much interest in the last decade.[5] The principle of the incorporation of a modified coenzyme analogue or a transition metal complex into a protein, was already developed by Kaiser[6] and Whitesides[7] in the late 1970's. In artificial transition metalloenzymes the catalytic activity stems mainly from the transition metal part, while the selectivity of the catalytic transformation is induced by the chiral environment of the protein.

To create structurally well-defined systems, site-selective functionalization of the host-protein with the organometallic catalyst is required. For covalent attachment, usually a suitably located highly reactive amino-acid functional group, like the nucleophilic thiol side-chain of a unique cysteine, is employed for anchoring of the catalyst.[5]

Phosphine ligands are an attractive class of ligands since the corresponding transition metal complexes are able to catalyze efficiently a variety of reactions such as olefin hydrogenation, hydroformylation and allylic substitution, reactions that are (as yet) not catalyzed by enzymes. In recent years several reports have been published on the successful non-covalent anchoring of phosphine ligands inside proteins[7–9] and antibodies[10] and the use of the resulting artificial metalloenzymes in asymmetric catalysis. The incorporation of phosphine ligands into the macromolecular host through a covalent linkage has been studied to a lesser extent, most likely due to the fact that most common methods for site-specific bioconjugation are unsuitable for phosphines. In 2002, Reetz and coworkers reported the covalent anchoring of a phosphine ligand within the active site of a lipase, but the obtained hybrid turned out to be hydrolytically unstable.[11]

Clearly, there is a need for reliable synthetic methodology for the covalent modification of proteins with a wide range of transition metal complexes. We recently demonstrated the first covalent, site-selective conjugation of free phosphines to a protein host providing a long term stable linkage.[12] Photoactive Yellow Protein (PYP) was site-selectively functionalised with phosphine-ligands and phosphine-palladium complexes, affording artificial metalloenzymes active in allylic substitution reactions. Here we show that the established conjugation method, employing phosphine-carboxylic acids, can also be applied to the site-selective coupling of rhodium-diphosphine complexes to the cysteine of PYP R52G, thus providing a platform for the synthesis of artificial transition-metalloenzymes for diverse catalytic transformations.

The strongly chelating properties of phenanthrolines for a large number of metals make it an attractive ligand for the synthesis of hybrid metalloproteins and metalloenzymes. Phenanthroline-modified proteins have been applied as synthetic metalloenzymes for DNA cleavage,[13] asymmetric ester and amide hydrolysis,[14] and Diels–Alder reactions.[15]

With applications in asymmetric transfer-hydrogenation and Diels–Alder reactions in mind, we also present here the

[a]*Van't Hoff Institute for Molecular Sciences, University of Amsterdam, Nieuwe Achtergracht 166, 1018 WV, Amsterdam, The Netherlands*
[b]*EaStCHEM, Department of Chemistry, University of St. Andrews, St. Andrews, Fife, KY16 9ST, United Kingdom. E-mail: pcjk@st-andrews.ac.uk, wwl1@st-andrews.ac.uk; Fax: +44-1334-463808*

† Electronic supplementary information (ESI) available: Details of protein production, MS spectra of conjugates and catalysis procedures. See DOI: 10.1039/c0dt00239a

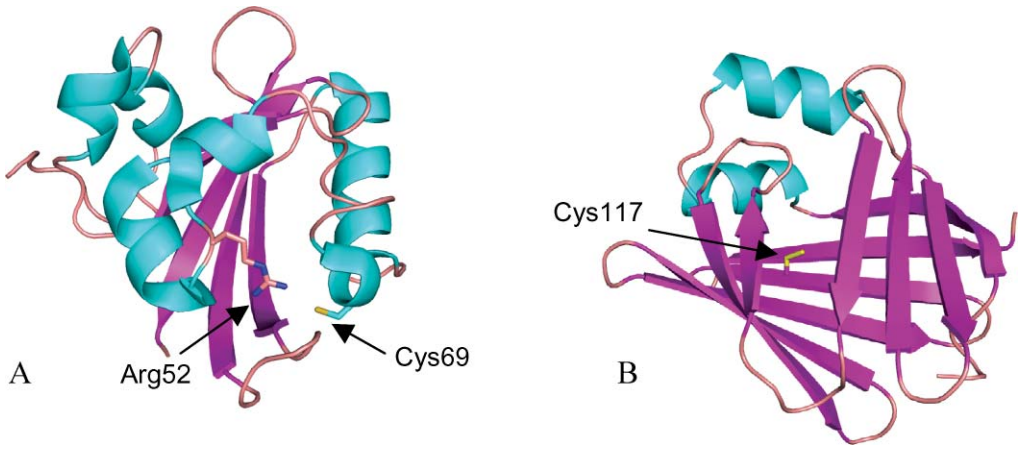


Fig. 1 Cartoon representation of (A) the crystal structure of the Photoactive Yellow Protein (PDB ID 2PHY) with Cys69 and Arg52 highlighted, and (B) ALBP (PDB ID 1LIB), with Cys117 highlighted.

preparation and characterization of novel metalloproteins obtained *via* the thiol-selective anchoring of a (η^6-arene) ruthenium(II) phenanthroline complex to PYP, PYP R52G and ALBP.

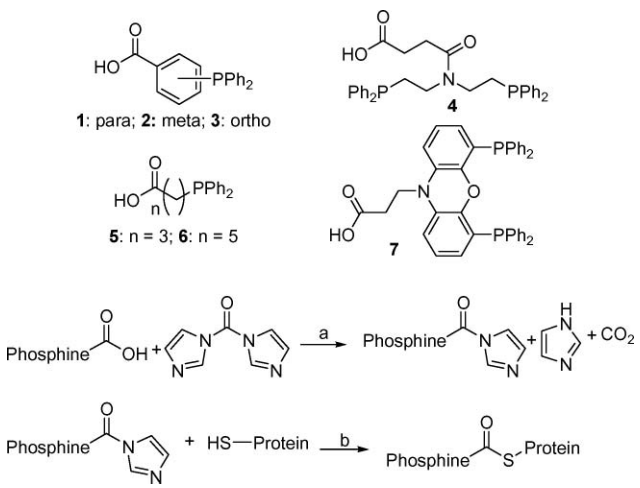
Results and Discussion

Among the different classes of proteins, transporters and enzymes are most frequently employed for the synthesis of artificial metalloenzymes. Our study has focused on the use of PYP as scaffold for the preparation of artificial metalloenzymes. PYP is a water-soluble, yellow photoreceptor protein, originally isolated from the bacterium *Halorhodospira halophila*.[16] By definition, photoreceptor proteins contain a light-absorbing chromophore, for PYP this is *p*-hydroxycinnamic acid.[17] In 1995, a crystal structure of the protein in the ground state was published (Fig. 1),[18] revealing two hydrophobic cores: the smaller of these is located at the N-terminus, while the larger contains the chromophore-binding pocket. In this pocket, the chromophore is covalently bound through a thiol ester linkage to the single cysteine of the protein, Cys69.[19,20]

In the mutant PYP R52G, the arginine at position 52 (Arg52) is replaced by a glycine. This is expected to result in a more accessible cavity, since Arg52 is believed to shield the chromophore from the solvent (Fig. 1). The highly nucleophilic thiol group of Cys69 in combination with the hydrophobic binding-pocket makes PYP an attractive scaffold for the covalent anchoring of transition metal complexes.

In PYP the chromophore is covalently bound through a thiol ester to Cys69. The apo-protein can be reconstituted *in vitro* with activated forms of the *p*-hydroxycinnamic acid chromophore; the use of the thiophenyl ester,[21] the anhydride[21] and the imidazolide[22] of *p*-hydroxycinnamic acid all lead to formation of the desired thioester linkage with the protein. Therefore we explored the use of carboxylic acid modified phosphine ligands for the site-selective covalent coupling of the phosphine moiety to the protein.

Because the only by-products of the activation of a carboxylic acid with *N*,*N*′-carbonyldiimidazole (CDI) and subsequent coupling reaction are imidazole and CO_2, which are easily removed from the protein after coupling, we chose to use CDI-activated phosphine-ligands for the protein-functionalization. The imidazolides of phosphino-carboxylic acids **1–7** were synthesised by reacting them with an excess of CDI in DMF (Scheme 1).[12] Phosphine ligands **1–7** (Scheme 1) are either commercial available or are readily obtained *via* short and efficient synthetic routes.



Scheme 1 Synthesis of phosphine-PYP conjugates *via* imidazolide formation of phosphine-carboxylic acids. (a) DMF, overnight, RT. (b) 50 mM Tris-HCl/H_2O, pH 8.0, overnight, RT.

The coupling of benzoic acid **3** with PYP R52G is described here in detail as an illustrative example. Compound **3** was activated with CDI in DMF and subsequently added to one equivalent of protein in buffered aqueous solution. The mixture was incubated overnight and the protein was purified by centrifugal ultrafiltration using a 10 kDa filter. LC-MS analysis of the purified protein demonstrated the clear presence of a PYP-adduct with a mass of 16,050 Da which perfectly matches the calculated mass of the conjugate of ligand **3** to R52G (Fig. 2). The successful coupling of benzoic acid **3** to R52G was also confirmed with ^{31}P NMR spectroscopy. The conjugate displayed a signal at $\delta = -4.8$ ppm which is typical of triphenylphosphine derivatives (Fig. 3). Using the same approach, ligands **1–7** have been site-selective covalently anchored to PYP R52G.[12] In all cases, the ionization efficiency during ESI-MS analysis of each hybrid was found to be about 80–100-fold less than that of the parent-protein, therefore revealing

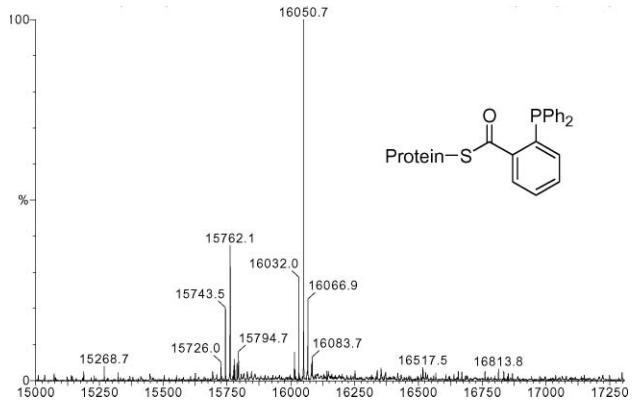


Fig. 2 ESI-MS analysis of PYP R52G modified with **3**.

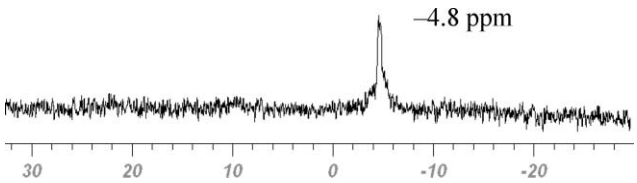


Fig. 3 ^{31}P NMR analysis of PYP R52G modified with **3**.

that all ligands coupled with more than 90% efficiency. The same was observed for the hybrids containing metal-complexes (see below).

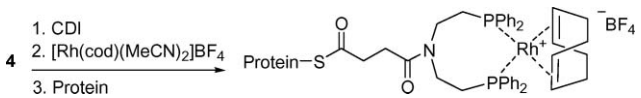
Analysis of the conjugates by trypsin digestion followed by MS analysis, failed to provide evidence for protein modification, probably due to the lability of the thioester under the trypsin digestion conditions. Instead, treatments with dithiothreitol (DTT) and alkaline conditions were performed on the new phosphine-containing proteins and the results indicated clearly that PYP R52G is modified with the phosphine ligands exclusively at Cys69.[12]

Another protein scaffold we are exploring is the adipocyte lipid binding protein (ALBP). ALBP is a small 131 residue protein with a simple architecture that mainly consists of two orthogonal planes of β-sheet secondary structure (Fig. 1). A variety of fatty acids bind in a large (600 Å^3) cavity formed between the two sheets. The suitability of this protein for our studies is exemplified by its previous successful application in the construction of artificial metalloenzymes, whereby organo(metallic) catalysts were introduced in the cavity by chemical modification of a cysteine.[14,23] Unfortunately, the phosphine-conjugation method developed for PYP lacked the desired chemoselectivity when applied to ALBP. The modification of lysines occurred in conjunction with thiol-modification, thus affording proteins containing multiple ligands (results not shown). A detailed account of this will be presented elsewhere (manuscript in preparation).

Rhodium conjugates

Next we focused on the preparation of artificial metalloenzymes, site-selectively modified with phosphine-rhodium complexes, for application in hydrogenation and hydroformylation reactions. From the start of the development of artificial metalloenzymes, the rhodium-catalyzed hydrogenation of alkenes has been intensively investigated. In 1978, Wilson and Whitesides reported the construction of a hydrogenation catalyst based on embedding an achiral biotin-functionalised rhodium-diphosphine moiety within the protein avidin.[7] In later studies, the groups of Chan[8] and in particular Ward[9] further developed this approach. In 2005, De Vries and coworkers showed that papain, covalently modified at Cys25 with a monodentate phosphite ligand and complexed with [Rh(cod)$_2$]BF$_4$, is an active catalyst in the hydrogenation of methyl α-acetamidoacrylate, but no enantioselectivity was observed.[24]

Inspired by these studies, we explored the challenging goal of building a covalently enzyme-bound rhodium-phosphine complex for hydrogenation reactions (Scheme 2). Reaction of the imidazolide of ligand **4** with [Rh(cod)(MeCN)$_2$]BF$_4$ afforded the corresponding chelating complex displaying two sets of double doublets in ^{31}P NMR spectroscopy at $\delta = 40$ and 45 ppm. This rhodium complex was successfully coupled to PYP R52G as the resulting conjugate showed a broad signal at $\delta = 36$ ppm in ^{31}P NMR spectroscopy (Figure S1†). It appears that in addition to LC-MS analysis, ^{31}P NMR spectroscopy is a useful technique for characterizing phosphorus-based artificial metalloenzymes.



Scheme 2 Synthesis of PYP R52G-[(**4**)Rh(cod)]BF$_4$.

The rhodium-catalyzed hydroformylation of alkenes using artificial metalloenzymes has been studied to a much lesser extent than the hydrogenation reaction despite the fact that hydroformylation is an interesting transformation since it cannot be achieved by natural enzymes. Using rhodium loaded human serum albumin (HSA), Marchetti, Paganelli and coworkers reported the hydroformylation of styrene and 1-octene under aqueous biphasic conditions.[25] For the hydroformylation of styrene good regioselectivity and high turnover numbers were obtained, but unfortunately, no mention of the enantioselectivity was made. Notably, the localization of the rhodium catalyst was not defined since no site-selective anchoring method was applied.

With the exception of this example, the Rh-catalyzed hydroformylation of alkenes using artificial metalloenzymes is still unexplored. We were interested in the synthesis of PYP-based hybrid catalysts for application in hydroformylation reactions through our developed cysteine-selective conjugation method. Reaction of the crude imidazolide of ligand **4** with [Rh(acac)(CO)$_2$] afforded a complex which showed two sets of double doublets in ^{31}P NMR spectroscopy at $\delta = 18$ and 19 ppm. Using the same procedure as for the conjugation of the free phosphines, this rhodium complex could also be selectively coupled to the protein, as revealed by LC-MS (Fig. 4). A species with a mass corresponding to the protein containing the ligand and rhodium(acac) was observed.

Ruthenium conjugates

Artificial metalloenzymes have been previously used in transfer hydrogenation of acetophenone and derivatives by Ward and coworkers.[27] In these studies the (strept)avidin and its mutants were conjugated with (η^6-arene) ruthenium(II) complexes modified with ethylenediamine biotinylated ligands (Noyori's catalyst) and applied successfully as transfer hydrogenases. Transfer hydrogenation reactions under aqueous conditions using cationic (η^6-arene) ruthenium phenanthroline molecular systems have been

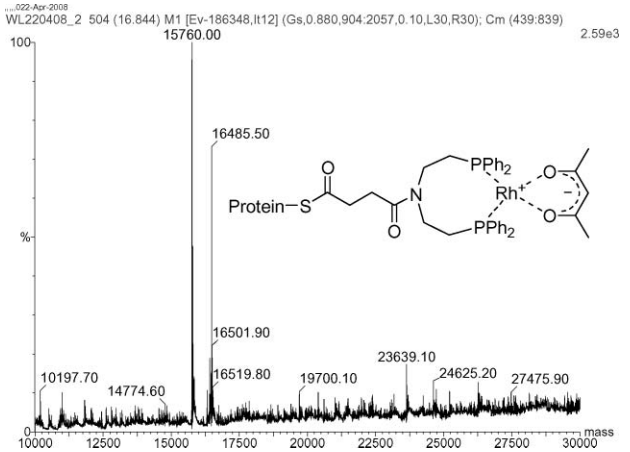
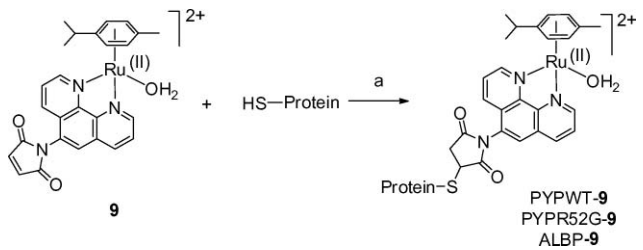


Fig. 4 ESI-MS analysis of PYP R52G modified with **4**-Rh(acac).

studied previously by Süss-Fink and co-workers.[28] Very recently Salmain *et al.* reported the application of papain modified with a phenantroline containing (η^6-arene) ruthenium(II) complex as an artificial Diels–Alderase.[15] Based on these precedents we decided to synthesise novel (η^6-arene) ruthenium(II) metalloproteins *via* thiol-selective conjugation of a maleimido-phenanthroline ruthenium complex.

The 5-maleimido-1,10-phenanthroline ligand can be easily synthesised from commercial reagents and obtained in good yields.[29] The corresponding dicationic *p*-cymene Ru(II) complex **9** was synthesised according to a procedure reported by Süss-Fink for similar phenanthrolines.[28]

The chemical modification of PYP WT, PYP R52G and ALBP with **9** was performed by stirring the protein and the ruthenium complex overnight in 20 mM Tris-HCl buffer at pH 7.0 (Scheme 3).



Scheme 3 Synthesis of Ru-phenanthroline-PYP and ALBP conjugates *via* thioether bond formation. (a) 20 mM Tris-HCl/H_2O, pH 7.0, overnight, RT.

The reaction of the ruthenium complex with PYP, PYP R52G and ALBP afforded in all cases the desired mono-modified protein and the amount of double coupling was negligible as revealed by LC-MS analysis (Fig. 5 and Figures S2 and S3†). Moreover, analysis of the relative ionisation efficiencies showed that the conversion was more than 90% for each protein. The LC-MS in Fig. 5 shows the signal corresponding to PYP modified with the dicationic fragment (*p*-cymene)Ru(OH_2)(Phen-maleimide)$^{2+}$ (16382.2 Da). The signal corresponding to the apo-protein appears at 15855.7 Da as well as a signal at 15887.6 Da, which we assign to the doubly oxidised protein. Similar results are obtained with PYP R52G. The doubly oxidised proteins probably arise from the oxidation of the methionine terminal group and/or the cysteine of

Table 1 Conjugation of **9** to PYP and ALBP

Entry	Protein	Conjugate	MW_{calcd}	MW_{obs}	ΔMW_{exp} (Δ_{cal} 528.5)
1	PYPWT	—	15861.8	15855.7	—
2	PYPWT	Ru(phen)	16390.3	16382.2	526.5
3	PYPR52G	—	15761.4	15758.5	—
4	PYPR52G	Ru(phen)	16289.9	16286.3	527.8
5	ALBP	—	16604.8	16599.8	
6	ALBP	Ru(phen)	17133.3	17126.2	526.4

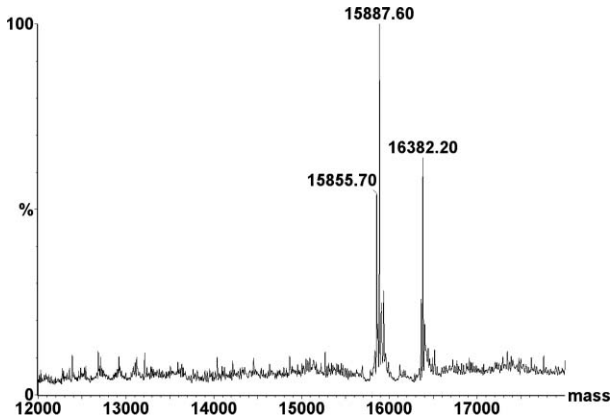


Fig. 5 ESI-MS analysis of PYP WT modified with Ru complex **9**.

PYP (WT and mutant) under the analysis conditions. In contrast, ALBP does not undergo any oxidation during the analysis.

Table 1 shows the main signals observed in the LC-MS. The difference of the main signal and the signal of the apo-proteins corresponds to the mass of the conjugation of **9**. The new ruthenoproteins were also characterised by MS of the peptide fragments obtained by trypsin digestion to confirm cysteine-selective bioconjugation.

For PYP WT-**9**, this analysis showed the signal corresponding to the unmodified cysteine containing peptide (1528.6 Da), which was also present in the protein solution. The signal corresponding to the modified peptide (2056.9 Da = 1528.6 + 528.3 Da) showed the expected pattern attributed to the ruthenium isotopes (Figure S4†), in agreement with the calculated mass spectrum of the modified peptide fragment containing the ruthenium complex (Figure S5†). Moreover, no other modified peptides were found. This confirms the introduction of the chemical modification on the cysteine containing peptide fragment. Similar analysis of PYP R52G-**9** and ALBP-**9** (Figure S6†) also confirmed thiol-selective anchoring in these proteins.

We further characterised PYP WT-**9** and ALBP-**9** by fluorescence spectroscopy. ALBP modified with 5-maleimido-1,10-phenanthroline (ALBP-Phen) exhibits an emission maximum at 420 nm, which is absent in ALBP and is attributed to the phenanthroline ligand. The phenanthroline emission is also absent in ALBP-**9,** showing that the binding of ruthenium leads to significant quenching of the phenanthroline fluorescence (Fig. 6A). In addition, in ALBP-Phen and particularly in ALBP-**9** the tryptophan fluorescence, centered around 330 nm, is reduced, likely due to quenching by the phenanthroline. These results are in good agreement with the results obtained by Distefano *et al.*, who observed similar fluorescence characteristics of ALBP modified with iodoacetamide-1,10-phenanthroline and copper [14a]

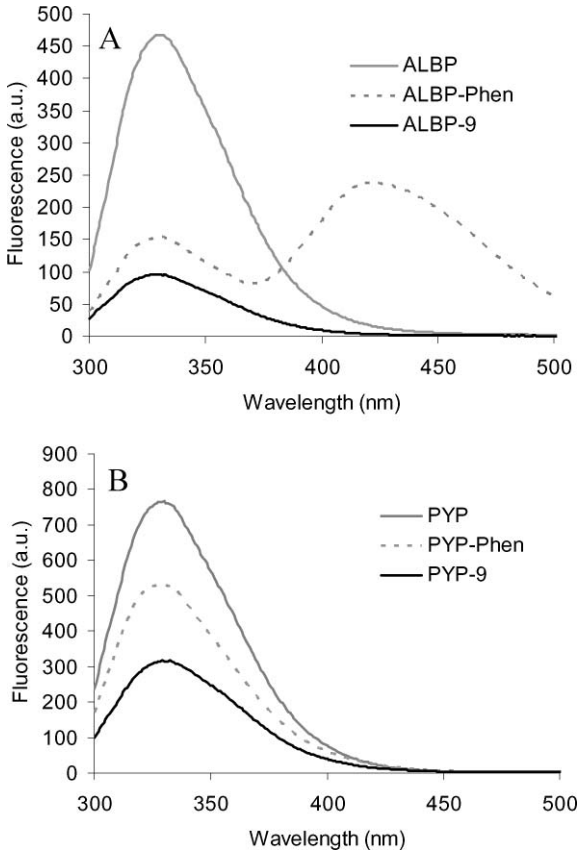


Fig. 6 (A) Fluorescence emission spectra of ALBP (grey line), ALBP-Phen (dashed line) and ALBP-**9** (black line) (B) Fluorescence emission spectra of PYP WT (grey line), PYP-Phen (dashed line) and PYP WT-**9** (black line).

and strongly suggest that the ruthenium is bound to the phenanthroline in ALBP-**9**. The relatively higher ratio of phenanthroline and tryptophan fluorescence observed in our system compared to that of Distefano may arise from more efficient quenching of the tryptophan fluorescence and/or an increase in phenanthroline fluorescence due to a more rigid anchoring of the maleimido-phenanthroline.

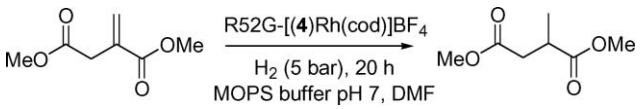
PYP WT modified with phenanthroline (PYP-Phen) does not show any discernable phenanthroline fluorescence, nor does PYP WT-**9**. Absence of phenanthroline fluorescence in phenanthroline-modified proteins has been reported previously.[14b] PYP-Phen and PYP-**9** do show reduced tryptophan fluorescence compared to PYP WT, but the phenanthroline induced quenching is less pronounced than that observed for ALBP (Fig. 6B).

Süss-Fink and coworkers have shown that using ruthenium(II) phenanthroline complexes as catalysts for transfer-hydrogenation, low pH and high temperatures favoured the formation of the alcohols.[28] Therefore we explored the temperature and pH stability of PYP WT-**9** and PYP R52G-**9**. In order to avoid denaturation of the new PYP metalloproteins the pH was adjusted to 6.5, in which both metalloproteins were found to be stable. The stability of the ruthenoproteins towards the temperature was studied using CD spectroscopy measurements in the near UV (250–310 nm) (Figures S7–9†). The temperature was varied from 20 to 80 °C, with increments of 10 °C and leaving the sample during an established time to equilibrate the temperature before each acquisition. The CD spectra corresponding to PYP WT modified with the ruthenium complex showed that the protein seems to keep its folded structure even at 50 °C. However, above 50 °C the CD spectra showed almost no signal suggesting the disappearance of the chiral environment. At the end of the experiment the protein solution contained a white precipitate indicating denaturation and further precipitation of the ruthenoprotein. Based on these results we decided to study the stability of the protein at 40 °C in time. The CD spectra obtained after three hours showed no significant changes in the spectra suggesting that the protein was sufficiently stable under these conditions. At the end of the experiment no protein precipitate was observed. Similar results were obtained with PYP R52G modified with the ruthenium complex. A similar characterization of ALBP-**9** is currently ongoing.

Catalysis

Following the successful application of the PYP-based palladium complexes in allylic amination reactions,[12] we performed preliminary catalysis studies with the here reported rhodium- and ruthenium-hybrid metalloproteins (see Supporting Information).

The R52G-[(**4**)Rh(cod)]BF_4 conjugate is an active catalyst for the hydrogenation of dimethyl itaconate (Scheme 4) although organic cosolvents were required to obtain reproducible conversions. We assume that under fully aqueous conditions the metal center in the R52G adducts are not accessible for substrates. The addition of organic solvents denatures the protein which results in a more accessible metal center. This also disrupts the tertiary structure of the protein which can explain the lack of induction of enantioselectivity.



Scheme 4 Hydrogenation of dimethyl itaconate.

The first experiments with the here reported PYP–Rh(acac) and PYP–Ru conjugates in hydroformylation and transfer hydrogenation reactions, respectively, were unsuccessful. The relatively high temperature, pressure of syngas or the low pH required for these reactions appear to be too harsh for the PYP-adducts. The ALBP conjugate has not been tested yet.

Conclusions

We have developed a general procedure for the site-selective covalent modification of PYP with phosphine ligands and their transition metal complexes. The thiol group of Cys69 in mutant R52G is highly reactive towards activated carboxylates. In this manner, carboxylate-containing phosphine ligands and their corresponding transition metal complexes were coupled to PYP R52G. The new artificial metalloproteins were characterised by LC-MS analysis and ^{31}P NMR spectroscopy, and one of the metalloproteins was found to be active in the rhodium-catalyzed hydrogenation in the presence of organic cosolvents.

Novel ruthenium metalloproteins were synthesised *via* thiol-selective coupling of a (η^6-arene) ruthenium(II) complex to PYP and ALBP; the PYP conjugate was inactive in the initial application in transfer-hydrogenation.

Experimental

General Procedures

Chemicals were purchased from Aldrich Chemical Co. and Fluka and were used as received. 5-maleimido-1,10-phenanthroline was prepared according to a literature procedure.[26] *N*,*N*-Dimethylformamide was dried over molecular sieves, degassed and stored under argon. THF was distilled from sodium/benzophenone, Silica gel 60 purchased from Fluka was used for column chromatography. All air- and water-sensitive reactions were carried out under dry, air free conditions using dry degassed solvents and standard Schlenk techniques under an atmosphere of purified argon. The conjugation of CDI-activated phosphine-ligands to PYP and PYP R52G was done following the procedure recently reported by our group.[12] NMR spectra were recorded at room temperature on a Bruker Avance II 400 spectrometer. ^{31}P NMR spectra of phosphine-modified proteins (1-2 mM samples, 26.000 scans, 10% D_2O as reference) were measured on a Bruker Avance 500. Positive chemical shifts (δ) are given (in ppm) for high-frequency shifts relative to a TMS reference (^{1}H and ^{13}C) or an 85% H_3PO_4 reference (^{31}P). ^{13}C and ^{31}P spectra were measured with ^{1}H decoupling. Coupling constants (J) are reported in Hz. Multiplicities are indicated by: s (singlet), d (doublet), dd (doublet of doublets), sept (septet) and m (multiplet). The abbreviation Ar is used to denote aromatic.

Synthesis of chloro-*p*-cymeme-(5-maleimido-1,10-phenanthroline) ruthenium(II) chloride [RuCl(phen-maleimide)(*p*-cymene)]Cl (8)

To a solution of $[RuCl_2(p\text{-cymene})]_2$ (95.8 mg, 0.156 mmol) in DCM (20 mL) was added phenanthroline-maleimide (56.4 mg, 0.32 mmol) and the solution was stirred during 3 h at room temperature. The solvent was removed under vacuum and the complex was obtained quantitatively and used without further purification in the next step. ^{1}H NMR (δ, $CDCl_3$, ppm): 0.96 (d, $J = 6.9$ Hz, 6H, *iso*-CH_3), 2.17 (s, 3H, CH_3), 2.8 (sept, $J = 6.9$ Hz, 1H, CH), 6.21 (d, $J = 6$ Hz, 1H, C_6H_4), 6.27 (d, $J = 6.2$ Hz, 1H, C_6H_4), 6.34 (d, $J = 6$ Hz, 1H, C_6H_4), 6.21 (d, $J = 6.2$ Hz, 1H, C_6H_4), 6.97 (s, 1H, CH maleimide), 6.98 (s, 1H, CH maleimide), 7.84 (s, 1H, CH= phen), 7.98 (m, 2H, CH= phen), 8.13 (d, $J = 8.3$, 1H, CH= phen), 8.46 (d, $J = 8.3$, 1H, CH= phen), 10.27 (d, $J = 4$, 2H, CH= phen).

Synthesis of aquo-*p*-cymene-(5-maleimido-1,10-phenanthroline) ruthenium(II) ditriflate, [Ru(OH_2)(phen-maleimide)-(*p*-cymene)](OSO_2CF_3)$_2$ (9)

The dichloro-p-cymene-(1,10)-phenanthroline-5-maleimide) ruthenium(II) complex (**8**) (152 mg, 0.32 mmol) was redisolved in distilled DCM (20 mL). The Schlenk vessel was covered with aluminium foil to avoid decomposition. Then, solid Ag(OTf) (180 mg, 0.70 mmol) was added to the solution and the reaction was stirred during 2 h at room temperature. The suspension (AgCl) was filtered over celite under argon and the clear filtrate was collected in a schlenk vessel. The solvent was removed under vacuum and the complex was obtained quantitatively as an orange solid. ^{1}H NMR (δ, D_2O, ppm): 0.82 (d, $J = 6.9$ Hz, 6H, *iso*-CH_3), 2.14 (s, 3H, CH_3), 2.45 (sept, $J = 6.9$ Hz, 1H, CH), 6.11 (d, $J = 6.5$ Hz, 2H, C_6H_4), 6.34 (d, $J = 6.5$ Hz, 2H, C_6H_4), 8.08 (dd, $J = 5.3$ Hz, $J = 8.3$ Hz, 2H, CH= phen), 8.13 (s, 2H, CH= phen), 8.80 (dd, $J = 1.2$, $J = 8.3$, 2H, CH= phen), 9.95 (dd, $J = 1.2$, $J = 5.3$, 2H, CH= phen). ^{13}C{^{1}H} NMR (δ, D_2O, ppm):17.6 (CH_3), 20.9 (CH(CH_3)$_2$), 30.4 (CH(CH_3)$_2$), 83.1 (C_6H_4), 86.5 (C_6H_4), 101.1 (C_6H_4), 103.7 (C_6H_4), 126.6 (CH= phen), 127.7 (CH= phen), 130.9 (C= phen), 140.3 (CH= phen), 146.0 (C= phen), 155.5 (CH= phen).^{19}F{^{1}H}NMR (δ, D_2O, ppm) −79.38 (CF_3SO_3). ESI-MS m/z (relative intensity): 528.7 (M^+).

General procedure for the conjugation of 9 to PYP and ALBP

In a Schlenk tube, 0.87 μmol of protein (1 eq.) in 10 mL 20 mM Tris-HCl, pH 7.0 was degassed by purging with argon for 4 h. While stirring, a solution of the maleimide ruthenium cationic complex (**9**) was added slowly (4.35 μmol, 5 eq., 87 μL of a 50 mM solution in distilled water for PYP, 10 eq. for ALBP). The clear reaction-mixture was stirred overnight at room-temperature for 16 h. The reaction-mixture was centrifuged at 49.000 g at 4 °C for 45 min to remove unreacted ligand. To remove imidazole and any remaining dissolved metal-complex, the yellow supernatant was transferred to a centrifugal concentrator (Millipore, MWCO = 10.000 Da) and concentrated to 500 μL followed by dilution with 20 mM Tris-HCl, pH 7.0. This was repeated four times, after which the modified protein was stored at 4 °C. (Final protein concentration is 960 μM). LC-MS analysis showed the formation of the desired conjugates (see supporting information†).

Fluorescence spectroscopy

Fluorescence measurements were performed at 25 °C using a Cary Eclips fluorescence spectrophotometer equipped with a Cary temperature controller. Fluorescence emission spectra were recorded from 300–500 nm after excitation at 275 nm (5 nm slits for both monochrometers, 100 nm min^{-1} scan speed) using protein solutions of 10 μM in 20 mM Tris-HCl, pH 7.0.

CD spectroscopy at variable temperature

Spectra were recorded on a Jasco, J-810 spectropolarimeter. Data in the near-UV (250–310 nm) were collected using a 0.5 mm path cuvette. The scan speed was 20 nm min^{-1}, with a bandwidth of 1 nm. Samples were prepared at 60 μM protein concentration in 20 mM Tris-HCl, pH 7.0. Each spectrum was measured six times, and the data were averaged to reduce the noise.

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 μM) 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 × 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 μL) 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 column and conditions: Supelco β-DEX 225 column ($T = 70\,^{\circ}C$ for 50 min, then $\Delta T = 25\,^{\circ}C\ min^{-1}$, t_R (S) = 51.7 min, t_R (R) = 52.3 min, t_R (substrate) = 53.5 min).

Acknowledgements

We thank Prof. Dr D. J. Cole-Hamilton for fruitful discussion. The plasmids and *E. coli* strain for expression of PYP and PYP R52G were kindly provided by Dr Michael van der Horst and Prof. Dr K. J. Hellingwerf, University of Amsterdam. A plasmid for expression of ALBP was kindly provided by Prof. D. Bernlohr and Dr B. Thompson, University of Minnesota. Financial support from the European Union is kindly acknowledged: NEST Adventure STREP Project artizymes contract No. FP6-2003-NEST-B3 15471; Marie Curie Excellence Grants, artizyme catalysis, contract number (MEXT-2004-014320), http://europa.eu.int/mariecurie-actions; Network of Excellence Idecat (Idecat-CT-2005-011730); COST action CM0802 PhoSciNet.

Notes and references

1 E. N. Jacobsen, A. Pfaltz and H. Yamamoto (ed.), *Comprehensive Asymmetric Catalysis*, Springer: Berlin, 1999.

2 P. W. N. M. van Leeuwen, *Homogeneous Catalysis, Understanding the Art*, Kluwer Academic Publishers: Dordrecht, 2004.

3 (*a*) K. Drauz and H. Waldmann (ed.), *Enzyme Catalysis in Organic Synthesis: Volumes I-III*, 2nd Edition, Wiley-VCH Verlag GmbH: Weinheim, 2002.; K. Faber, *Biotransformations in Organic Chemistry: a textbook*, 4th Edition, Springer-Verlag: Berlin, Heidelberg, 2000.

4 (*a*) M. T. Reetz, A. Zonta, K. Schimossek, K. Liebeton and K.-E. Jaeger, *Angew. Chem., Int. Ed. Engl.*, 1997, **36**, 2830; (*b*) M. T. Reetz and K.-E. Jaeger, *Chem.–Eur. J.*, 2000, **6**, 407; (*c*) M. T. Reetz, *Pure Appl. Chem.*, 2000, **72**, 1615.

5 For recent reviews on artificial metalloenzymes, see: (*a*) J. Steinreiber and T. R. Ward, *Coord. Chem. Rev.*, 2008, **252**, 751; (*b*) T. Ueno, S. Abe, N. Yokoi and Y. Watanabe, *Coord. Chem. Rev.*, 2007, **251**, 2717; (*c*) Y. Lu, *Angew. Chem., Int. Ed.*, 2006, **45**, 5588; (*d*) C. Letondor and T. R. Ward, *ChemBioChem*, 2006, **7**, 1845; (*e*) C. M. Thomas and T. R. Ward, *Chem. Soc. Rev.*, 2005, **34**, 337.

6 (*a*) H. L. Levine and E. T. Kaiser, *J. Am. Chem. Soc.*, 1978, **100**, 7670; (*b*) H. L. Levine, Y. Nakagawa and E. T. Kaiser, *Biochem. Biophys. Res. Commun.*, 1977, **76**, 64.

7 (*a*) O. Abril and G. M. Whitesides, *J. Am. Chem. Soc.*, 1982, **104**, 1552; (*b*) M. E. Wilson and G. M. Whitesides, *J. Am. Chem. Soc.*, 1978, **100**, 306.

8 C. C. Lin, C. W. Lin and A. S. C. Chan, *Tetrahedron: Asymmetry*, 1999, **10**, 1887.

9 (*a*) J. Collot, J. Gradinaru, N. Humbert, M. Skander, A. Zocchi and T. R. Ward, *J. Am. Chem. Soc.*, 2003, **125**, 9030; (*b*) M. Skander, N. Humbert, J. Collot, J. Gradinaru, G. Klein, A. Loosli, J. Sauser, A. Zocchi, F. Gilardoni and T. R. Ward, *J. Am. Chem. Soc.*, 2004, **126**, 14411; (*c*) J. Collot, N. Humbert, M. Skander, G. Klein and T. R. Ward, *J. Organomet. Chem.*, 2004, **689**, 4868; (*d*) U. E. Rusbandi, C. Lo, M. Skander, A. Ivanova, M. Creus, N. Humbert and T. R. Ward, *Adv. Synth. Catal.*, 2007, **349**, 1923; (*e*) U. E. Rusbandi, M. Skander, A. Ivanova, C. Malan and T. R. Ward, *C. R. Chimie*, 2007, **10**, 678.

10 H. Yamaguchi, T. Hirano, H. Kiminami, D. Taura and A. Harada, *Org. Biomol. Chem.*, 2006, **4**, 3571.

11 M. T. Reetz, M. Rentzsch, A. Pletsch and M. Maywald, *Chimia*, 2002, **56**, 721.

12 W. Laan, B. K. Muñoz, R. den Heeten and P. C. J. Kamer, *ChemBioChem*, 2010, **11**, 1236.

13 C. B. Chen, L. Milne, R. Landgraf, D. M. Perrin and D. S. Sigman, *ChemBioChem*, 2001, **2**, 735.

14 (*a*) R. R. Davies and M. D. Distefano, *J. Am. Chem. Soc.*, 1997, **119**, 11643; (*b*) R. R. Davies, H. Kuang, D. Qi, A. Mazhary, E. Mayaan and M. D. Distefano, *Bioorg. Med. Chem. Lett.*, 1999, **9**, 79.

15 B. Talbi, P. Haquette, A. Martel, F. de Montigny, C. Fosse, S. Cordier, T. Roisnel, G. Jaouen and M. Salmain, *Dalton Trans.*, 2010, **39**, 5605.

16 (*a*) T. E. Meyer, *Biochim. Biophys. Acta, Bioenerg.*, 1985, **806**, 175; (*b*) T. E. Meyer, E. Yakali, M. A. Cusanovich and G. Tollin, *Biochemistry*, 1987, **26**, 418.

17 W. D. Hoff, P. Dux, K. Hard, B. Devreese, I. M. Nugteren-Roodzant, W. Crielaard, R. Boelens, R. Kaptein, J. van Beeumen and K. J. Hellingwerf, *Biochemistry*, 1994, **33**, 13959.

18 G. E. Borgstahl, D. R. Williams and E. D. Getzoff, *Biochemistry*, 1995, **34**, 6278.

19 J. J. van Beeumen, B. V. Devreese, S. M. vanBun, W. D. Hoff, K. J. Hellingwerf, T. E. Meyer, D. E. McRee and M. E. Cusanovich, *Protein Sci.*, 1993, **2**, 1114.

20 (*a*) W. D. Hoff, B. Devreese, R. Fokkens, I. M. Nugteren-Roodzant, J. van Beeumen, N. Nibbering and K. J. Hellingwerf, *Biochemistry*, 1996, **35**, 1274.

21 Y. Imamoto, T. Ito, M. Kataoka and F. Tokunaga, *FEBS Lett.*, 1995, **374**, 157.

22 U. K. Genick, S. Devanathan, T. E. Meyer, I. L. Canestrelli, E. Williams, M. A. Cusanovich, G. Tollin and E. D. Getzoff, *Biochemistry*, 1997, **36**, 8.

23 H. Kuang, M. L. Brown, R. R. Davies, E. C. Young and M. D. Distefano, *J. Am. Chem. Soc.*, 1996, **118**, 10702.

24 (*a*) L. Panella, J. Broos, J. Jin, M. W. Fraaije, D. B. Janssen, M. Jeronimus-Stratingh, B. L. Feringa, A. J. Minnaard and J. G. de Vries, *Chem. Commun.*, 2005, 5656; (*b*) J. G. de Vries and L. Lefort, *Chem.–Eur. J.*, 2006, **12**, 4722.

25 (*a*) C. Bertucci, C. Botteghi, D. Giunta, M. Marchetti and S. Paganelli, *Adv. Synth. Catal.*, 2002, **344**, 556; (*b*) M. Marchetti, G. Mangano, S. Paganelli and C. Botteghi, *Tetrahedron Lett.*, 2000, **41**, 3717.

26 (*a*) R. W. Eckl, T. Priermeier and W. A. Herrmann, *J. Organomet. Chem.*, 1997, **532**, 243; (*b*) M. D. Miquel-Serrano, A. M. Masdeu-Bultó, C. Claver and D. Sinou, *J. Mol. Catal. A: Chem.*, 1999, **143**, 49; (*c*) J. A. J. Breuzard, M. L. Tommasino, M. C. Bonnet and M. Lemaire, *J. Organomet. Chem.*, 2000, **616**, 37.

27 (*a*) C. Letondor, N. Humbert and T. R. Ward, *Proc. Natl. Acad. Sci. U. S. A.*, 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.

28 J. Canivet, G. Karmazin-Brelot and G. Süss-Fink, *J. Organomet. Chem.*, 2005, **690**, 3202.

29 (*a*) M. T. Reetz, M. Rentzsch, A. Pletsch, A. Taglieber, F. Hollmann, R. J. G. Mondière, N. Dickmann, B. Höcker, S. Cerrone, M. C. Haeger and R. Sterner, *ChemBioChem*, 2008, **9**, 552; (*b*) S. A. Trammell, H. M. Goldston, Jr., P. T. Tran, L. M. Tender, D. W. Conrad, D. E. Benson and H. W. Hellinga, *Bioconjugate Chem.*, 2001, **12**, 643.