DOI: 10.1002/cbic.201000159

Artificial Metalloenzymes through Cysteine-Selective Conjugation of Phosphines to Photoactive Yellow Protein

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

The embedding of organometallic catalysts into the chiral environment of proteins and DNA to develop enantioselective hybrid transition metal catalysts is attracting increasing attention.[1] Phosphine ligands are ubiquitous in transition metal chemistry and can afford extremely reactive and versatile homogeneous catalysts. Consequently, various efforts have been made to create hybrid catalysts with this attractive class of ligands. The covalent embedding of phosphine ligands into DNA[2] and the application of phosphine-functionalized DNA in asymmetric catalysis has recently been described.[3] So far, protein-based artificial metalloenzymes containing phosphine ligands have mainly been developed by using noncovalent anchoring strategies. The introduction of biotinylated phosphines to (strept)avidin has been a particularly successful approach for the development of enantioselective artificial metalloenzymes.[4] Furthermore, the potential of using antibodies for the development of hybrid catalysts through supramolecular anchoring of phosphine catalysts has recently been demonstrated.[5]

In contrast, no examples of artificial metalloenzymes based on robust covalent phosphine conjugation to a protein have been reported to date, and so the protein structure space combined with this class of ligand remains limited. Nevertheless, Reetz has modified the active-site serine of a number of lipases with a diphosphine coupled to a phosphonate inhibitor, but unfortunately the resulting hybrids turned out to be hydrolytically unstable, which hampered application in catalysis.[6]

The unique reactivity of the nucleophilic thiol side chain of cysteine makes it a very attractive target for site-selective bioconjugation to proteins, which has previously been used for the covalent anchoring of synthetic catalysts. However, because of the nucleophilic character of phosphines, the most common methods for cysteine-selective bioconjugation, such as, disulfide bridge formation or alkylation by using haloacetamides and maleimides are incompatible with phosphines. Thus, alternative strategies need to be explored for this class of ligand. De Vries et al. turned to less-nucleophilic phosphites that could be covalently attached to papain. This resulted in an active, but nonselective hydrogenation catalyst.[7] Following a different approach, we have developed for the first time the site-selective covalent conjugation of phosphine ligands and phosphine–transition-metal complexes to a protein, and we report the application of some of the hybrids in catalysis.

Photoactive yellow protein (PYP) is a small (15 kDa) water-soluble photoreceptor protein from the bacterium *Halorhodospira halophila* (Figure 1).[8] The protein has a strong absorbance peak at 446 nm due to its chromophore, *p*-hydroxycinnamic acid, which is located in a small hydrophobic binding pocket and covalently linked by a thioester linkage to the unique cysteine, Cys69, of the protein.[9] The (recombinant) PYP apo-protein can be reconstituted in vitro with activated forms of the *p*-hydroxycinnamic acid chromophore or other chromophore derivatives: the use of the thiophenyl ester, the anhydride and the imidazolide of *p*-hydroxycinnamic acid can all lead to highly efficient and selective formation of the desired thioester linkage with the protein.[10]

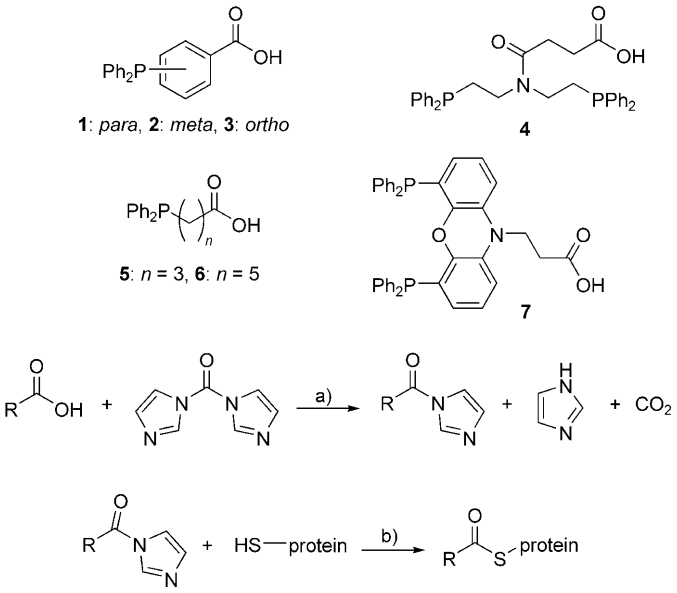
We decided to explore whether this reconstitution approach could be adopted for the site-selective coupling of phosphine ligands to the cysteine of PYP by using phosphino-carboxylic acids. The only by-products of the activation of a carboxylic acid with *N*,*N*-carbonyldiimidazole (CDI) to form the reactive imidazolide and subsequent coupling reaction are imidazole and CO_2. Because they 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 treating them with an excess of *N*,*N*-carbonyldiimidazole (CDI) in DMF (Scheme 1). For all ligands, a shift of the signal in the ^{31}P NMR spectrum occurs upon imidazolide formation, thus allowing the extent of activation to be monitored by NMR.

Treatment of the PYP with imidazolides of **1**–**7** afforded in all cases the desired conjugate in high yield and with excellent chemoselectivity. The predominant LC-MS signal found for each conjugate corresponds to PYP containing free phosphine (Table 1 and Figures S1–S3 in the Supporting Information). While the bidentate ligands **4** and **7** were the least reactive, the use of a larger excess still afforded excellent conversion. The lower reactivity might be due to the increased steric hindrance encountered by these bulkier bidentate ligands or a faster rate of hydrolysis of the imidazolide. Due to their nucleophilicity, free phosphines react similarly to free thiols with reagents used for the colorimetric detection of thiol groups. This prohibited the use of such assays to determine the specificity and efficiency of the coupling reactions. Instead, we relied on mass spectrometry to determine the extent of modification. Although in most cases a signal corresponding to the unmodified protein was still observed, the ionization efficiency of each hybrid was found to be about 80–100-fold less than that of the parent-protein, therefore revealing that all ligands coupled with more than 90% efficiency (see the Supporting Informa-

[a] Dr. W. Laan, Dr. B. K. Muñoz, Prof. Dr. P. C. J. Kamer
School of Chemistry, University of St Andrews
North Haugh, KY16 9ST, St. Andrews (UK)
Fax: (+44) 1334463808
E-mail: pcjk@st-andrews.ac.uk

[b] Dr. R. den Heeten
Van 't Hoff Institute for Molecular Sciences, University of Amsterdam
Nieuwe Achtergracht 166, 1018 WV Amsterdam (NL)

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/cbic.201000159.



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

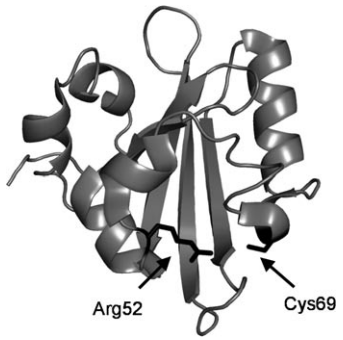


Figure 1. Crystal structure of PYP (PDB ID: 2PHY).[9a] The cysteine employed for bioconjugation and the arginine flanking the binding pocket are highlighted.

tion). The amount of double coupling observed was negligible for all ligands.

To confirm that the conjugates were exclusively formed by thioester formation, conjugates were incubated either with dithiothreitol (DTT) or at pH 14, the conditions that result in cleavage of the thioester linkage in native PYP.[9b,c] Both treatments result in complete disappearance of the LC-MS signal corresponding to the hybrids (Figures S6–S9), thus confirming the chemoselective conjugation of the ligands by a thioester bond to the unique cysteine of PYP. Even though the use of imidazolides is a classical way to modify proteins through lysine acylation, the lysines of PYP appear to be sufficiently unreactive at pH 8 to prevent any competitive amine modification. Furthermore, analysis of conjugates stored at 4 °C at neutral pH for several weeks revealed the linkage to be stable over prolonged periods under these conditions.

Table 1. Conjugation of phosphines **1**–**7** to PYP WT and PYP R52G.

Protein	Ligand	Conversion [%][a]	$M_{W\,calcd}$	$M_{W\,obs}$
WT	1	>90	16150.1	16150.0
WT	2	>90	16150.1	16149.9
WT	3	>90	16150.1	16151.1
WT	4	>90	16386.0	16385.6
WT	5	>90	16116.9	16117.1
WT	6	>90	16144.9	16143.4
WT	7	>90	16468.4	16466.9
R52G	1	>90	16050.7	16050.3
R52G	2	>90	16050.7	16048.8
R52G	3	>90	16050.7	16052.9
R52G	4	>90	16286.6	16287.8

[a] Estimated from relative LC-MS signal intensities.

In holo-PYP, Arg52 shields the binding pocket from the solvent.[11] Substituting a glycine for this residue is expected to lead to increased accessibility and could afford artificial metalloenzymes with different activity and selectivity. Similar conjugation results were indeed obtained when the mutant protein PYP R52G was used, thus indicating that the conjugation methodology is not restricted to the wild-type (WT) protein. Further analysis of selected conjugates by $^{31}P\{^1H\}$ NMR spectroscopy confirmed the introduction of the phosphine ligands as free phosphines; in each case, a relatively broad peak with a chemical shift typical for the respective free phosphine was found. For example, PYP WT-**1** displayed a signal at $\delta = -6.3$ ppm (Figure 2, top), while PYP R52G-**4** showed a signal at $\delta = -20$ ppm (Figure S4), these are typical values for free triphenylphosphine derivatives. To the best of our knowledge, this is the first demonstration of covalent, site-selective conjugation of free phosphines to a protein host providing a long-term stable linkage.

Next, we explored the potential of the established conjugation method for the synthesis of catalytic palladium-containing hybrids. Proteins contain numerous potential donor sites for palladium, as was clearly shown by the coordination of palladi-

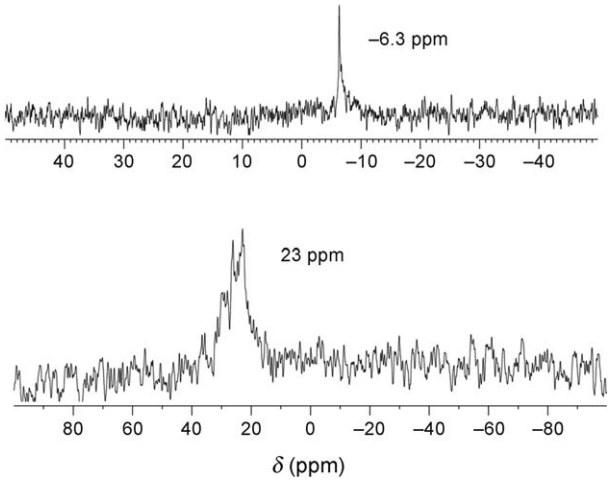
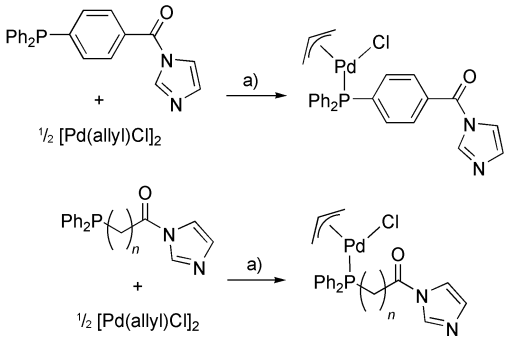


Figure 2. $^{31}P\{^1H\}$ NMR analysis of phosphine-modified PYP. Top: PYP WT modified with **1**; bottom: PYP R52G modified with **1**-Pd(allyl)Cl.

um–allyl moieties at multiple sites of ferritin.[12] To ensure discrete coordination of the transition metal to the phosphine in the presence of the alternative donor atoms of proteins,[12] we conjugated preformed palladium complexes of some of the ligands. Treatment of crude imidazolides of ligands **1, 5** and **6** with $[Pd(allyl)Cl]_2$ (Scheme 2) afforded yellow complexes that



Scheme 2. Formation of palladium–allyl complexes of the imidazolides of ligands **1** (top), **5** ($n=3$) and **6** ($n=5$, bottom). a) DMF, RT, 30 min.

exhibited broad signals around $\delta = 23$ ppm in ^{31}P NMR spectra, which are typical values for this type of phosphine–palladium complex. By using the same procedure for the conjugation of the free phosphines, these palladium complexes could also be selectively and efficiently coupled to the protein, as revealed by LC-MS (Figures 3, S10 and S11). In each case, a species with

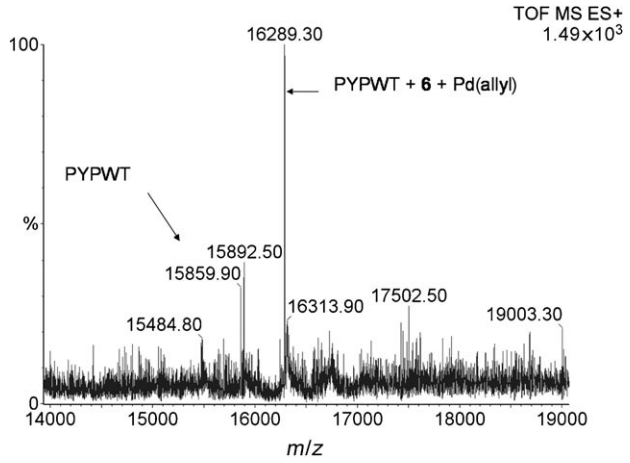
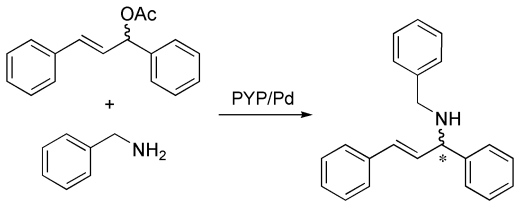


Figure 3. ESI-MS analysis of PYP WT modified with **6**-Pd(allyl)Cl.

a mass corresponding to the protein containing the ligand and palladium–allyl was observed. The apparent loss of the chloride either occurs during the LC-MS analysis procedure, or this ligand is displaced by an amino acid upon formation of the conjugates. To verify that the procedure affords conjugates in which the palladium is still coordinated to the phosphine ligand, R52G modified with **1**-Pd(allyl)Cl was characterized by ^{31}P NMR spectroscopy. The conjugate exhibits a broad signal at $\delta = 23$ ppm, (Figure 2, bottom) almost identical to the shift found for the unconjugated complex, thus confirming that palladium remains bound to the phosphine ligand. Analysis of the metalloproteins by circular dichroism (CD) spectroscopy revealed that the introduction of the synthetic catalysts has no significant effect on the structure of the protein (Figure S13).

Allylic substitution is a synthetically important catalytic reaction for which significant asymmetric induction has been achieved with various transition-metal-modified macromolecules.[13–16] The PYP-based hybrid metalloproteins were evaluated in palladium-catalysed allylic amination, by employing the model substrate 1,3-diphenylprop-2-enyl acetate and benzylamine as the nucleophile (Scheme 3 and Table 2). When the reactions were performed in aqueous buffered solution, irreproducible conversion was observed, this was presumably due to solubility problems with the substrate and/or inaccessibility of the metal centre. When the reactions were performed in a mixture of DMF and buffered aqueous solution (1:1), all metalloproteins gave good and reproducible conversions to the amination product, and hydrolysis of the substrate was negligible. Unfortunately, no enantiomeric excess was obtained. This could result from (partial) denaturation of the protein due to the high concentration of organic solvent (the structures of PYP and PYP R52G unfold above 5% DMF, as determined by CD, data not shown) causing the metal site to be too far removed from the chiral environment of the protein. No conversion was observed when the reaction was performed with unmodified protein in the presence of palladium precursor, thus showing that the observed activity indeed stems from phosphine coordinated palladium. Unfortunately, the synthesis of palladium complexes of ligands **2**–**4** proved to be difficult to reproduce. In some cases, red complexes were obtained that did not couple to the proteins, this is presumably due to the formation of palladium black.



Scheme 3. Asymmetric allylic amination: addition of benzylamine to 1,3-diphenylprop-2-enyl acetate.

Table 2. Allylic substitution of 1,3-diphenyl-2-propenyl acetate with benzylamine catalysed by phosphine-palladium modified PYP.[a]

Protein	Ligand	Conversion [%]	Protein	Ligand	Conversion [%]
WT	1	70	WT	6	88
WT	5	53	R52G	1	90

[a] Reaction conditions: 40 nmol PYP-L-Pd, benzylamine/ propenyl acetate/Pd = 110:50:1, 50 mM bicine, pH 8.5, 50% DMF, 0.5 cm^3, 25 °C, 24 h.

In summary, we have for the first time established a method for the cysteine-selective bioconjugation of phosphines and

have used this method to synthesise artificial metalloenzymes. We are currently exploring the use of more water-soluble substrates for the allylic amination reactions that would obviate the need for organic cosolvents and the concomitant protein denaturation. Furthermore, the established conjugation method can be applied to the coupling of rhodium–phosphine complexes (unpublished results), thus providing a platform for the synthesis of phosphine-based artificial transition metalloenzymes for diverse catalytic transformations.

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). This research was financed by the NRSCC and the European Union: NEST Adventure STREP Project artizymes contract No. FP6-2003-NEST-B3 15471; Marie Curie Excellence Grants, artizyme catalysis, contract no. (MEXT-2004-014320), http://europa.eu.int/mariecurie-actions.*

Keywords: bioconjugation · catalysis · hybrid catalysts · metalloenzymes · transition metals

[1] a) J. Steinreiber, T. R. Ward, *Coord. Chem. Rev.* **2008**, *252*, 751 – 766; b) T. Ueno, S. Abe, N. Yokoi, Y. Watanabe, *Coord. Chem. Rev.* **2007**, *251*, 2717 – 2731.

[2] M. Caprioara, R. Fiammengo, M. Engeser, A. Jaschke, *Chem. Eur. J.* **2007**, *13*, 2089 – 2095.

[3] L. Ropartz, N. J. Meeuwenoord, G. A. van der Marel, P. W. N. M. van Leeuwen, A. M. Slawin, P. C. J. Kamer, *Chem. Commun.* **2007**, 1556 – 1558.

[4] a) M. E. Wilson, G. M. Whitesides, *J. Am. Chem. Soc.* **1978**, *100*, 306 – 307; b) J. Collot, J. Gradinaru, N. Humbert, M. Skander, A. Zocchi, T. R. Ward, *J. Am. Chem. Soc.* **2003**, *125*, 9030 – 9031; c) M. T. Reetz, J. J. Peyralans, A. Maichele, Y. Fu, M. Maywald, *Chem. Commun.* **2006**, 4318 – 4320; d) C. C. Lin, C. W. Lin, A. S. C. Chan, *Tetrahedron Asymmetry* **1999**, *10*, 1887 – 1893.

[5] H. Yamaguchi, T. Hirano, H. Kiminami, D. Taura, A. Harada, *Org. Biomol. Chem.* **2006**, *4*, 3571 – 3573.

[6] M. T. Reetz, M. Rentzsch, A. Pletsch, M. Maywald, *Chimia* **2002**, *56*, 721 – 723.

[7] L. Panella, J. Broos, J. F. Jin, M. W. Fraaije, D. B. Janssen, M. Jeronimus-Stratingh, B. L. Feringa, A. J. Minnaard, J. G. de Vries, *Chem. Commun.* **2005**, 5656 – 5658.

[8] T. E. Meyer, E. Yakali, M. A. Cusanovich, G. Tollin, *Biochemistry* **1987**, *26*, 418 – 423.

[9] a) G. E. Borgstahl, D. R. Williams, E. D. Getzoff, *Biochemistry* **1995**, *34*, 6278 – 6287; b) J. J. Van Beeumen, B. V. Devreese, S. M. Vanbun, W. D. Hoff, K. J. Hellingwerf, T. E. Meyer, D. E. Mcree, M. A. Cusanovich, *Protein Sci.* **1993**, *2*, 1114 – 1125; c) W. D. Hoff, P. Dux, K. Hard, B. Devreese, I. M. Nugteren-Roodzant, W. Crielaard, R. Boelens, R. Kaptein, J. Van Beeumen, K. J. Hellingwerf, *Biochemistry* **1994**, *33*, 13959 – 13962.

[10] a) Y. Imamoto, T. Ito, M. Kataoka, F. Tokunaga, *FEBS Lett.* **1995**, *374*, 157 – 160; b) U. K. Genick, S. Devanathan, T. E. Meyer, I. L. Canestrelli, E. Williams, M. A. Cusanovich, G. Tollin, E. D. Getzoff, *Biochemistry* **1997**, *36*, 8 – 14.

[11] U. K. Genick, G. E. O. Borgstahl, K. Ng, Z. Ren, C. Pradervand, P. M. Burke, V. Šrajer, T. Teng, W. Schildkamp, D. E. McRee, K. Moffat, E. D. Getzoff, *Science* **1997**, *275*, 1471 – 1475.

[12] S. Abe, J. Niemeyer, M. Abe, Y. Takezawa, T. Ueno, T. Hikage, G. Erker, Y. Watanabe, *J. Am. Chem. Soc.* **2008**, *130*, 10512 – 10514.

[13] P. Fournier, R. Fiammengo, A. Jäschke, *Angew. Chem.* **2009**, *121*, 4490 – 4493; *Angew. Chem. Int. Ed.* **2009**, *48*, 4426 – 4429.

[14] J. Pierron, C. Malan, M. Creus, J. Gradinaru, I. Hafner, A. Ivanova, A. Sardo, T. R. Ward, *Angew. Chem.* **2008**, *120*, 713 – 717; *Angew. Chem. Int. Ed.* **2008**, *47*, 701 – 705.

[15] C. A. Christensen, M. Meldal, *Chem. Eur. J.* **2005**, *11*, 4121 – 4131.

[16] A. Agarkov, S. J. Greenfield, T. Ohishi, S. E. Collibee, S. R. Gilbertson, *J. Org. Chem.* **2004**, *69*, 8077 – 8085.

Received: March 10, 2010
Published online on April 29, 2010

