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Artificial Metalloenzymes in Asymmetric Catalysis. Key Developments and Future Directions.

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Abstract. Artificial metalloenzymes combine the excellent selective recognition/binding properties of enzymes with transition metal catalysts, and therefore many asymmetric transformations can benefit from these entities. The search for new successful strategies in the construction of metal-enzyme hybrid catalysts has therefore become a very active area of research. This review discusses all the developed strategies and the latest advances in the synthesis and application in asymmetric catalysis of artificial metalloenzy mes with future directions for their design, synthesis and application (Sections 2-4). Finally, advice is presented (to the non-specialist) how to prepare and use artificial metalloenzymes (Section 5).

Keywords: artificial metalloenzymes; asymmetric catalysis; hybrid catalysts

1 Introduction

Nowadays most of the high-value compounds, such as pharmaceuticals, fragrances, and agrochemicals, as well as many of the chemicals used in functional materials, are required as pure enantiomers.[1] As a result, the industrial production of enantiopure chiral compounds is gaining importance and synthetic procedures are constantly evolving towards high selectivity, atom economy, operational simplicity, cost efficiency, environmental friendliness, and low energy consumption. In comparison to other synthetic approaches, asymmetric catalysis is a smart strategy. A small amount of catalyst can produce large quantities of the desired chiral compound with only a few reaction steps and synthetic operations, thus bringing down the overall production cost, and decreasing the amount of byproducts.

Enzymes have excellent selective recognition properties, so they usually provide the highest levels of selectivity. However, many important fine chemicals are produced by homogeneous metal catalysis because the efficient enzymes needed for a given transformation are lacking (i.e. C-C bond forming reactions). In this context in recent years considerable efforts have been devoted to the development of metal-enzyme hybrid catalysts (artificial metalloenzymes) for enantioselective organic transformations.[2-3] This approach combines the excellent selective recognition/binding properties of enzymes with transition metal catalysts. Combining biological concepts of selective recognition with those of metal-catalysis has led to the development of new highly selective hybrid catalysts for several asymmetric catalytic reactions. Moreover, anchoring the metal-catalyst in the host enzyme increases its biocompatibility and therefore facilitates its recyclability, while maintaining the best advantages of metal-catalyst. The optimization of the artificial metalloenzymes for a given reaction is aided by directed evolution and combinatorial techniques.

Inspired by the pioneering work of Kaiser[4] and Whitesides[5], the search for new successful strategies in the construction of metal-enzyme hybrid catalysts has become a very active area and the focus of many important research groups. Only in recent years a large number of new and impressive results have been reported with the development of new approaches that still need to be optimized. In this context, remarkable efforts have been made to extend the scope of bioconjugation strategies to increase the specific binding of the metal fragment, as well as, to enlarge the variety of ligands and metals that can be incorporated into the enzyme. A review that discusses all the developed strategies and latest advance under

the same perspective, providing a global overview of the research done and the possibilities for future research is crucial to speed up its future success. This review therefore offers a critical overview of the limitations of the methodologies used up to now, together with examples of recent alternatives developed in the artificial metalloenzyme design to overcome these limitations.

The review is organized as follows. In section 2 we present the methodologies used to redesign natural occurring metalloenzymes, as well as its application in asymmetric catalysis. In section 3, we review the strategies used to generate artificial metalloenzymes from metal-free enzymes and their application in asymmetric catalysis. This section has been organized according to the three main different approaches used to anchor transition metals into native metal-free enzymes (namely direct metal salt complexation, covalent anchoring and the non-covalent/supramolecular anchoring of the metal complex). In Section 4, we cover recent reports on the use of hybrid catalysts with metalloenzyme-like properties that maintain the enzymatic catalytic activity. In Section 5, advice is presented (to the non-specialist) how to prepare and use artificial metalloenzymes. Finally, a section with conclusions and future perspectives is also included.

Oscar Pàmies obtained his Ph.D. in Prof. Carmen Claver's group in 1999 at the Rovira i Virgili University. After three years of post-doctoral work in the group of Prof. J.-E. Bäckvall at the Department of Organic Chemistry at Stockholm University, he returned to Tarragona in 2002. He is currently working as associate professor at the Rovira i Virgili University. He received the Grant for Research Intensification from URV in 2008. Recently he has been awarded the ICREA Academia Prize 2010 from the Catalan Institution for Research and Advanced Studies. His research interests are asymmetric catalysis, organometallic chemistry and combinatorial synthesis.

Montserrat Diéguez studied chemistry at the Rovira i Virgili University in Tarragona (Spain), where she received her Ph.D. in 1997 working in the group of Prof. C. Claver. After a year as postdoctoral fellow with Prof. R.H. Crabtree at Yale University, in New Haven (USA), she returned to Tarragona in 1999 and accepted a lecturer position at the University Rovira i Virgili, becoming part of the permanent staff in 2002. In 2011 she was promoted to full Professor in Inorganic Chemistry at the University Rovira i Virgili in Tarragona. She has been involved in more than 30 research projects in the field of organometallic chemistry, steroselective synthesis and asymmetric catalysis. She is author of more than 100 articles in SCI indexed Journals and book chapters and of several contributions to Conferences. She obtained the Distinction from the Generalitat de Catalunya for the promotion of University Research in 2004 and the Grant for Research Intensification from URV in 2008. Recently she has been awarded the ICREA Academia Prize 2009 from the Catalan Institution for Research and Advanced Studies. Her main research interests are focused on the sustainable design, synthesis and screening of highly active and selective chiral catalysts for reactions of interest in the biological, pharmaceutical and organic nanotechnological industry. Her areas of interest include organometallic chemistry, steroselective synthesis and asymmetric catalysis using combinatorial and biotechnological approaches.

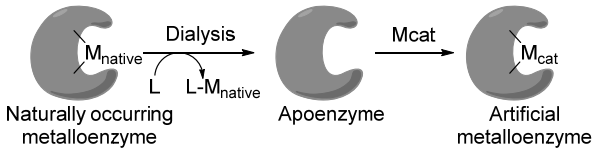
Jan-Erling Bäckvall was born in Malung, Sweden, in 1947. He received his Ph.D. from the Royal Institute of Technology, Stockholm, in 1975 with Prof. B. Åkermark. After post-doctoral work (1975 76) with Prof. K. B. Sharpless at Massachusetts Institute of Technology he joined the faculty at the Royal Institute of Technology. He was appointed Professor of Organic Chemistry at Uppsala University in 1986. In 1997 he moved to Stockholm University where he is currently Professor of Organic Chemistry. He is a member of the Royal Swedish Academy of Sciences, Finnish Academy of Science and Letters, and Academia Europaea. He is a member of the Nobel Committee for Chemistry. He is a member of a number of Editorial Boards of journals and he is the Chairman of the Editorial Board of Chemistry – A European Journal. He has edited the book "Modern Oxidation Methods" Wiley-VCH, 2004, 2nd edition 2010. He has been visiting Professor in Lyon (France), Rennes (France), Utrecht University (the Netherlands), Strasbourg (France), Alicante (Spain), Paris (France), Mülheim (Germany), and Caltech, Pasadena (USA). He recently (2014) became honorary doctor at Åbo Akademi University, Finland. His current research interests include transition metal-catalyzed organic transformations, biomimetic oxidations, and enzyme catalysis.

2 Redesigning naturally occurring metalloenzymes

Metalloenzymes constitute almost a third of all enzymes found in nature. The variety of metals incorporated and the inherent diversity in their primary, secondary and tertiary structure makes them applicable to many different chemical processes. Metalloenzymes containing metals such as Fe, Cu, Zn, and Mn are known to have specific functions or to catalyze specific reaction.[6] Fe-containing cytochromes such as cytochrome P450 are known to catalyze oxidation of C-H bonds whereas other Fe-containing enzymes such as hemoglobin transport molecular oxygen from the lungs to the tissues. Other examples include naturally occurring nitrogenases (Fe and Mo) that catalyze the fixation of atmospheric nitrogen,[7] hydrogenases (Fe and Ni) that are involved in the reversible oxidation of molecular hydrogen,[8] halo-peroxidases (V) that are able to catalyze the

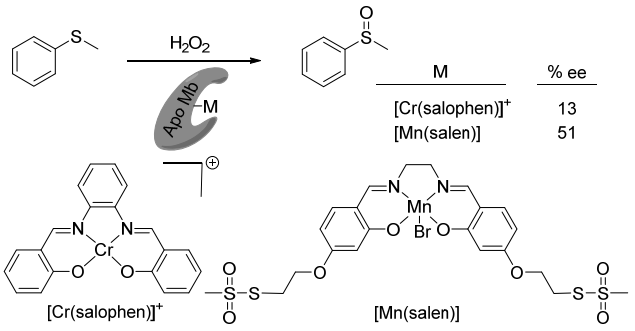
asymmetric epoxidation and dihydroxylation of olefins as well as the asymmetric sulfoxidation of thioethers.[9]

Up to now, three main procedures for redesigning/reconstructing a naturally occurring metalloenzyme for a given catalytic reaction have been developed. The first approach involves the replacement of the native metal by a suitable transition metal. The main requirement is that the apoenzyme (enzyme without the native metal) must be stable to prevent degradation during the metal exchange. Although, it is possible to express the enzyme without the native metal, most of the examples make use of dialysis against metal chelating species to generate the desired apoenzyme (Scheme 1).



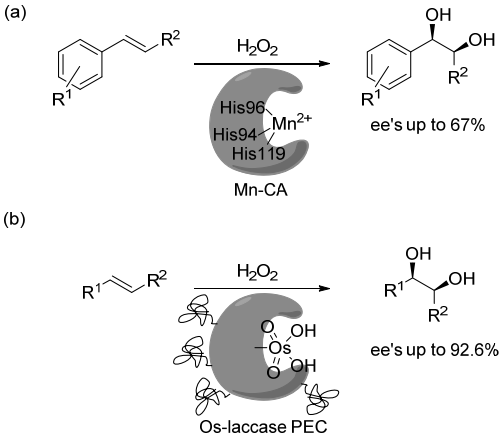
Scheme 1. Redesigning naturally occurring metalloenzymes by replacement of the native metal by a suitable transition metal.

Kaiser and coworkers were the first to demonstrate that replacement of the native Zn(II) in carboxypeptidase A (CPA) by Cu(II), via dialysis with 1,10-phenantroline,[10] modifies its catalytic activity.[4] The new metalloenzyme Cu(II)-CPA, which lacks the peptidase and sterease activities of the native CPA, is able to oxidize ascorbic acid. In 2003, Watanabe and coworkers were able to incorporate [MIII(salophen)]+ (M= Mn, Cr) complexes into the chiral cavity of apo-myoglobin (apo-Mb).[11] The insertion takes place by binding of the metal complexes to His93 of the active site. In order to increase the binding affinity of the [Cr(salophen]$^+$ complex in the chiral active site, they designed a suitable mutant by replacing Ala71 by Gly (A71G). As a result the new metalloenzyme is able to catalyze the enantioselective sulfoxidation of thioanisole, albeit with low reactivity and enantioselectivity (ee's up to 13%). The low enantioselectivity achieved suggests that multiple orientations of the [M^{III}(salophen)]$^+$ complex may be possible in the active site. In an attempt to address this drawback, Lu and coworkers used a dual anchoring strategy to limit the rotational freedom of the metal complex.[12] Thus, they designed a Mn-salen complex with pendant methanethiosulfonate groups and modified the apo-Mb to place two cysteines at positions L76C and Y103C to favor the selective covalent anchoring of the Mn(salen) complex into the apo-Mb. This dual anchoring strategy not only increased the enantioselectivities (up to 51%), but also increased the reaction rates (Scheme 2).



Scheme 2. Asymmetric sulfoxidation of thioanisole using artificial M-Mb metalloenzymes.

Carbonic anhydrases (CA) is another class of naturally occurring metalloenzymes in which the native Zn^{2+} has been replaced by other transition metals. The corresponding apo-carbonic anhydrase (apo-CA) has been successfully prepared by dialysis with 2,6-pyridinecarboxylate. The first examples include the preparation of Mn-containing metalloenzymes for enantioselective epoxidation of olefins (Scheme 3a).[13] Although the affinity of CA for manganese is low, the epoxidation activity of Mn-CA was higher than that of free manganese, so the observed epoxides are formed from the reaction of Mn-CA catalyst. The enantioselectivity in the epoxidation of styrenes exhibited by Mn-CA was moderate (ee's up to 67%), but comparable to or better than that of natural peroxidases.[14]



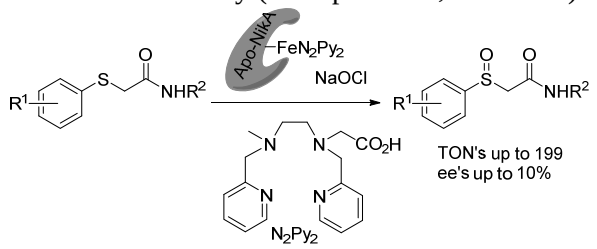
Scheme 3. Asymmetric dihydroxylation of alkenes using (a) Mn-CA metalloenzyme; and (b) conjugate of poly(2-methyloxazoline) and Os-laccase metalloenzyme (Os-laccase PEC).

More recently, Kazlauska's group has successfully replaced the active site zinc in CA with rhodium complexes ([$Rh(cod)_2$]BF_4 and [$Rh(acac)(CO)_2$]).[15] However, they found that rhodium also bound to the side chains of the histidine residues on the enzyme surface. Removal of these histidine residues by chemical modification and/or site-directed mutagenesis minimized the nonspecific binding of rhodium to the surface. The resulting Rh-CA

metalloenzymes were applied in the regioselective hydroformylation of styrene[15b] and the chemoselective hydrogenation of *cis*-stilbene.[15a] It should be pointed out that in in the the hydroformylation of styrene, rhodium bound to the active site of CA favors the formation of the linear aldehyde product up to 8.4 fold over the branched product.[15b]

More recently, the group of Tiller and coworkers have prepared conjugates of copper-type I laccase from *Trametes vesicolor* and poly(2-methyl-oxazoline), which are soluble in organic media.[16] Replacement of the native copper by osmate, using EDTA as Cu-scavenger, led to the formation of a new organo-soluble metalloenzyme (Os-laccase PEC) that is active in the dihydroxylation of a range of alkenes (ee's between 0% to 92.6%; Scheme 3b).

Recently, Ménage and coworkers took advantage of the apo-NikA's (nickel-binding protein) ability to bind carboxylate Fe-N_2Py_2 complexes (such as Fe-EDTA) through supramolecular interactions to prepare new metalloenzymes.[17] The new metalloenzymes were efficient in the sulfoxidation of aryl thioglycolamides (TON's up to 199), albeit with poor enantioselectivity (ee's up to 10%; Scheme 4).

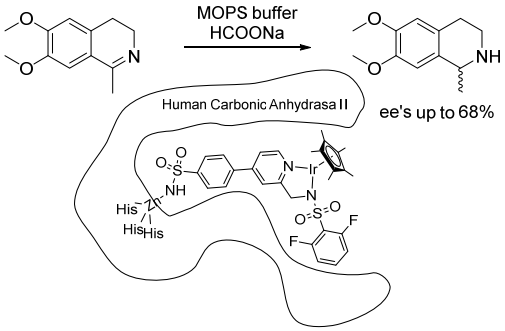


Scheme 4. Asymmetric sulfoxidation of aryl thioglycolamides using artificial Fe-N_2Py_2-apo-NikA.

Ferritin (Fr) is an iron storage protein formed by 24 subunits that resembles a hollow cagelike structure, with a cavity of diameter 8 nm. Small channels, which are crucial for the transport of small molecules, are located at the junctions of the subunits. Interestingly, apo-ferritin can be easily recombinantly expressed and reconstituted with a range of non native metals as Watanabe and coworkers have recently demonstrated. They first prepared a zero-valent Pd cluster inside the ferritin cavity (Pd^0-Fr).[18] The new Pd^0-Fr metalloenzyme was effective in the size-selective olefin hydrogenation in aqueous media. The same group was also able to bind [Pd(η^3-allyl)Cl] dimer on the ferritin interior (Pd_{allyl}-Fr).[19] Two distinct binding sites were detected, one at the 3-fold axis channel and the other in the so-called "accumulation center". Although site-directed mutagenesis was performed to control the binding sites, the efficiency of the Pdallyl-Fr in the Suzuki coupling of phenylboronic acid and 4-iodoaniline demonstrates that the binding site control was not completed. Similarly, they also showed that [Rh(nbd)$(Cl_2)]_2$ complex efficiently bind to apo-Fr (Rh-Fr). Although, the XR analysis of Rh-Fr showed three different binding sites for the Rh(nbd) complex, the Rh-Fr efficiently catalyzes the polymerization of phenylacetylene with restricted molecular weight (MW) and a narrow MW distribution.[20]

In summary, with very few exceptions the replacement of native metal by a suitable transition metal provided poor-to-moderate enantioselectivities. A plausible explanation can be found in the presence of multiple metal binding sites in the apo-enzyme, which led to the formation of mixtures of catalytic species with different chiral environments within the unnatural metalloenzyme.

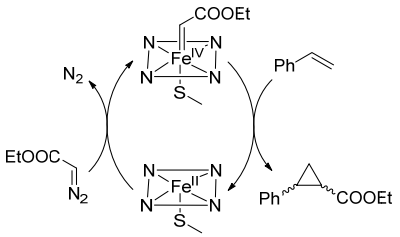
The second strategy for redesigning a naturally occurring metalloenzyme consists in using the natural metalloenzyme as a host for creating a new bimetallic metalloenzyme. In this approach a new transition metal complex is bound directly to the native metal via a common ligand linker. In this way this approach overcomes the drawback of the presence of mixtures of active catalytic centers of the first approach. Ward and coworkers were the first to illustrate this approach. For this purpose they took advantage of the high affinity of the *para*-substituted arylsulfonamides to the catalytic Zn anion. Thus, they tethered an aryl-sulfonamide to a bidentated N-N ligand to anchor an Ir-Cp* catalysts within a human Carbonic Anhydrase II to form an artificial transfer hydrogenase (Ir-hCAII; Scheme 5).[21] They found that incorporation of the [IrCp*(X)Cl] in hCAII efficiently reduces 6,7-dimethoxy-1-methyl-3,4-dihydroisoquinoline under transfer hydrogenation conditions in good enantioselectivity (ee's up to 68%). However, severe substrate inhibition was observed when using the wild type hCAII. This drawback was solved by replacing the close-lying isoleucine 91 into an alanine (I91A). This new optimized metalloenzyme Ir-I91A-hCAII displayed significant rate enhancement over the organometallic catalyst. Further enantioselectivity improvement can be easily envisaged by additional engineering the catalytic site of hCAII either by chemical modification or by site-directed mutagenesis.



Scheme 5. Asymmetric transfer hydrogenation of 6,7-dimethoxy-1-methyl-3,4-dihydroisoquinoline using optimized metalloenzyme Ir-I91A-hCAII

The third strategy consists in modifying the natural metalloenzyme, while maintaining the native metal, to perform important reactions not previously

observed in nature. Arnold and coworkers demonstrated that this approach is valid.[22] Thus, by protein engineering of the cytochrome P450 from *Bacillus megaterium* (P450BM3), containing a heme group in the enzyme active site, they were able to perform a highly diastereo- and enantioselective cyclopropanation of styrenes from diazoesters via carbene transfer (Scheme 6). Thus, mutant $P450_{BM3}$-T268A afforded almost exclusively the *trans* cyclopropane in 96% ee. Interestingly, they also found that by further site-saturation mutagenesis it is possible to reverse regioselectivity towards the *cis* product, while maintaining the excellent enantioselectivities. Thus, variant $P450_{BM3}$-CIS-T438S afforded the *cis* product in a ratio 92:8 and 97% ee.



Metalloenzyme	*cis/trans*	ee_{cis}	ee_{trans}
$P450_{BM3}$-T268A	1/99	-15	-96
$P450_{BM3}$-CIS-T438S	92/8	-97	-66

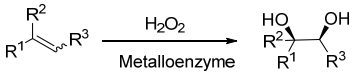
Scheme 6. Artificial mode of artificial cytochrome P450s for the stereoselective cyclopropanation of styrene.

3 Creation of new metalloenzymes from metal-free enzymes

Three different approaches have been developed to anchor transition metals to native metal-free enzymes to form hybrid artificial metalloenzymes: (i) direct metal salt complexation, (ii) covalent anchoring of the metal complex and (iii) the non-covalent/supramolecular anchoring.

3.1 Artifical metalloenzymes via direct metal salt complexation

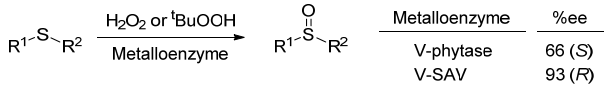
In this approach a metal salt or complex binds to the metal-free enzyme by using specific amino acids (binding sites) to form a new hybrid catalyst. The first successful application of this approach in asymmetric catalysis was reported in 1983 by Kokubo et al.[23] They were able to anchor OsO_4 in bovine serum albumin (BSA) through lysine residues present in the BSA. The hybrid catalyst Os-BSA catalyzed the *cis*-dihydroxylation of alkenes with ee's up to 68% ee (Scheme 7). More recently, Ward and coworkers improved the enantioselectivity up to 77% ee by using $K_2[OsO_2(OH)_4]$ instead of OsO_4 and slightly different reaction conditions.[24] In the same study they disclosed that by using wild-type streptavidin (SAV) instead of BSA, activities and enantioselectivities improved (TON's up to 27 and ee's up to 95%; Scheme 7). The enantioselectivity could be improved to 97% ee by using mutant SAV-S112Y (Scheme 7).[24] X-Ray analysis of Os-SAV metalloenzyme indicated multiple osmium binding sites. Three of them, which are located in the proximity of the chiral pocket, were subjected to site-directed mutagenesis. The corresponding Os-SAV mutants provided similar levels of enantioselectivity as those achieved with the wild-type. This suggests that not all the osmium incorporated in SAV is catalytically active.



Metalloenzyme	TON	%ee
Os-BSA	4	68 (*S*)
Os-SAV	27	95 (*R*)
Os-S112YSAV	16	97 (*R*)

Scheme 7. Asymmetric dihydroxylation of alkenes using osmium artificial metalloenzymes generated via direct metal salt complexation.

Vanadium salts have also been directly incorporated into enzymes that do not naturally bind metals. In the late 90's, Sheldon and coworkers reported the oxidation of thioethers by H_2O_2 catalyzed by vanadium-incorporated phytase (from *Aspergillus ficcum*). At low vanadium concentrations, they achieved full conversions to the corresponding sulfoxide with enantioselectivities up to 66% ee (Scheme 8).[25] It has been proposed that 5 different amino acid residues (Arg_{490}, Arg_{360}, His_{404}, His_{496} and Lys_{353}) are involved in the binding of the oxovanadate species in the phytase.[25b] In 2008, Ward and coworkers incorporated $VOSO_4$ into SAV.[26] The new hybrid V-SAV catalyzes enantioselective oxidation of aryl/alkyl- and dialkylsulfides in up to 93% ee (Scheme 8). Characterization and mutagenesis studies suggest that the vanadyl ion is located in the biotin-binding pocket and that the second coordination sphere of SAV influences enantioselectivity.

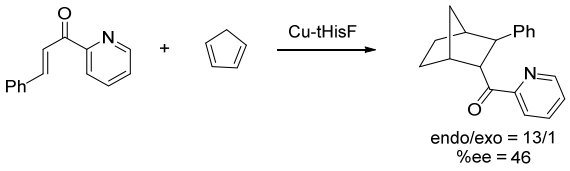


Scheme 8. Asymmetric sulfoxidation of thioethers using vanadium artificial metalloenzymes generated via direct metal salt complexation.

Marchetti and coworkers developed a series of new artificial metalloenzymes by incorporating $[Rh(CO)_2(acac)]$ complex to several albumins such as human serum albumin (HSA) and egg albumin[27] The combined of Rh-HSA catalyst turned out to be extremely effective in the aqueous biphasic hydroformylation of styrene with substrate-to-catalysts ratios as high as 700000:1 at moderate pressure (10 bar CO/H_2) and low temperature (40 ^{0}C) with good regioselectivities (up to 90% towards the

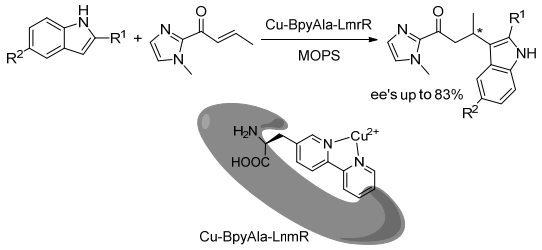
branched product). The high efficiency has been attributed to the protecting environment conferred by the HSA to the Rh centers towards O_2 and other poisons[28]. However, catalytic data indicated that multiple nonspecific binding sites are present, which may have a detrimental effect on enantioselectivity (no enantioselectivity data were reported).

As previously shown, the presence of multiple possible binding sites in enzymes that do not naturally bind metals is one of the main limitations of this approach if one seeks excellent levels of enantioselectivity. To overcome this drawback, different approaches to generate specific binding sites into the enzymes have been studied. Thus for instance, Reetz and coworkers created a specific Cu-binding site in a thermostable enzyme from *Thermotoga maritima* (tHisF).[29] They were inspired by nature's design found in the type-2 copper proteins, in which the Cu is coordinated by two nitrogens of the imidazole group of histidine and one or more oxygen residues. They therefore engineered a 2-His-1-carboxylate motif (His/His/Asp) at the top of the TIM barrel of tHisF. Replacement of the Cys9 and the remaining histidine residues in the tHisF (located at positions 84, 209, 228 and 244) by Ala was further necessary, because cysteine and histidine residues are known to compete for the metal binding. The resulting mutant was metalated with $CuSO_4$ and applied in the Cu-catalyzed enantioselective Diels-Alder reaction. The resulting mutant Cu-tHisF provided notable enantioselectivity (ee's up to 46%) and high *endo* selectivity (*endo*/*exo* = 13:1) in the Diels-Alder cycloaddition of azachalcone and cyclopentadiene (Scheme 9).



Scheme 9. Asymmetric Diels-Alder reaction using a metalloenzyme which was modified to generate a specific copper binding site.

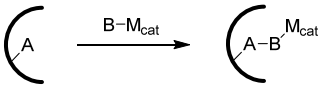
Another interesting alternative for the generation of specific metal binding sites is the introduction via mutagenesis of unnatural amino acids with strong metal binding properties. Thus, Schultz and coworkers incorporated the unnatural amino acid (2,2'-bipyrimidin-5-yl)alanine (BpyAla)[30] and (8-hydroxyquinolin-3-yl)alanine (HQAla)[31] into several proteins. Although these amino acids strongly bind Cu and Zn species, the generated proteins have not been applied to asymmetric catalysis. Roelfes and coworkers have recently prepared artificial Cu-metalloenzymes with an in vivo incorporation of metal-binding unnatural amino acid BpyAla into the protein LmrR, using the stop codon suppression methodology.[32] The resulting artificial Cu-BpyAla-LmrR metalloenzymes catalyzed the asymmetric Friedel-Crafts alkylation reactions with enantioselectivities as high as 83% ee (Scheme 10). Mutagenesis studies clearly showed the importance of the second coordination sphere for the product outcome.



Scheme 10. Asymmetric Friedel-Crafts alkylation using Cu-BpyAla-LmrR metalloenzymes with a binding site generated by introduction of an unnatural amino acid.

3.2 Artifical metalloenzymes via covalent anchoring

In this strategy a ligand of a catalytically active transition metal complex containing an specific linker is covalently bound to a specific amino acid residue of the enzyme (Scheme 11). Kaiser and coworkers in the late 70's settled the basis for the generation of artifical enzymes via covalent modification. They therefore demonstrated that bromomethyl- and bromoacetyl-flavins can be linked to the active site cysteine of papain to generate artificial flavopapain oxidoreductasas.[33]

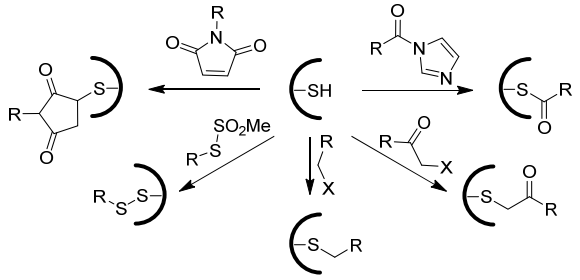


Scheme 11. Representative formation of an artificial metalloenzyme via covalent interaction.

For the preparation of an artificial metalloenzyme using this approach there are two basic requirements that have to be fulfilled: The first one is that the enzyme must contain a single reactive amino acid residue. The second requirement is that the enzyme must contain a chiral cavity big enough to accommodate the metal moiety, the reactants and the substrate. The presence of a single reactive residue to which the metal complex can bind is necessary to avoid the anchor of several catalytically active species within the enzyme, which will hamper the stereochemical control of the reaction outcome. Knowledge of the structure of the enzyme is therefore crucial. For example, the X-ray structures of many of the hydrolases have shown that they contain a single serine or cysteine residue that can be used to covalently link the metal complex,[34] which makes the use of this enzyme class very appealing. It should be pointed out that enzymes containing multiple active binding residues can also be used, but it is necessary to remove the extra binding sites prior to the covalent metal linkage. However, the generation

of the appropriate mutants (apart from being time consuming) may be problematic if the residues to be mutated play an important role for the stability and structure of the enzyme.

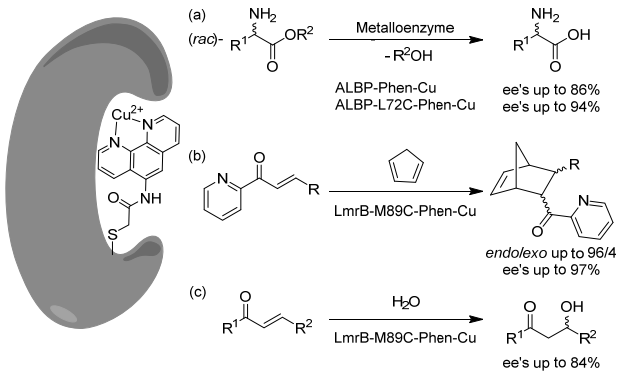
Most of the artificial metalloenzymes make use of cysteine as a residue to covalently link the metal complex. This can be attributed to the great variety of selective coupling reactions (bioconjugation) with cysteine that have been developed. The cysteine residue can be easily modified using for examples α-haloacetyls, maleimides or imidazolides (Scheme 12). In addition cysteine mutations (to install or remove cysteine residues) can be readily introduced into the enzyme by site-directed mutagenesis.



Scheme 12. Representative bioconjugated methods for cysteine residues.

Diestefano and coworkers were the first to apply artificial metalloenzymes, created via a covalent interaction, in asymmetric catalysis.[35] They used iodoacetamido-1,10-phenantroline to modify the unique cysteine residue in adipocyte lipid binding protein (ALBP) and the resulting modified protein was used as a template to bind Cu^{2+}. The new copper metalloenzyme (ALBP-Phen-Cu) catalyzed the enantioselective hydrolysis of several unactivated amino acid esters under mild reaction conditions with moderate-to-high enantioselectivities (ee's up to 86%; Scheme 13a). Soon after, the same group was able to increase enantioselectivity (up to 94%; Scheme 13a) by positioning the copper-phenantroline catalyst at a different location within the protein cavity (mutation ALBP-L72C).[36]

More recently, Roelfes and coworkers also created artificial metalloenzymes by covalent anchoring Cu(II)-phenantroline complex but in the hydrophobic pocket of the LmrR scaffold.[37] Since no natural cysteine residues are present in LmrR, they prepared LmrR N19C and M89C mutants by site-directed mutagenesis. The positions 19 and 89 were selected on the basis of the X-ray structure of LmrR and computer modelling. The artificial metalloenzyme LmrB-M89C-Phen-Cu provided excellent enantio-selectivities in the Diels-Alder reaction (ee's up to 97%; Scheme 13b)[37a] as well as in the challenging 1,4-addition of water to α,β-unsaturated ketones (ee's up to 87%; Scheme 13c)[37b].



Scheme 13. Representative applications of Cu(II)-phenantroline-based artificial metalloenzymes in asymmetric catalysis. Enantioselective (a) hydrolysis of unactivated amino acid esters, (b) Diels-Alder reaction and (c) 1,4-addition of water to α,β-unsaturated ketones.

Other complexes containing nitrogen-donor ligands have also been covalently bound to cysteine residues.[38-40] These include the bioconjugation to papain of Rh- and Ru-half sandwich complexes containing bidentated nitrogen ligands by Samain's group (Figure 1a).[39] The new artificial metalloenzymes catalyzed Diels-Alder reactions and transfer hydrogenation of ketones in water albeit with low enantioselectivity (up to 15% ee). More recently, Kamer's group developed artificial copper metalloenzymes from sterol carrier protein type 2 like domain (SCP-2L) in which the newly generated cysteine residues were coupled with various nitrogen ligands.[40] Maleimide-containing ligands were found to be most suitable for cysteine bioconjugation. They found that metalloenzyme SCP-2L-V83C-Cu was able to catalyze Diels-Alder reactions with a moderate *endo*/*exo* ratio and a low enantioselectivity (up to 25% ee; Figure 1b).

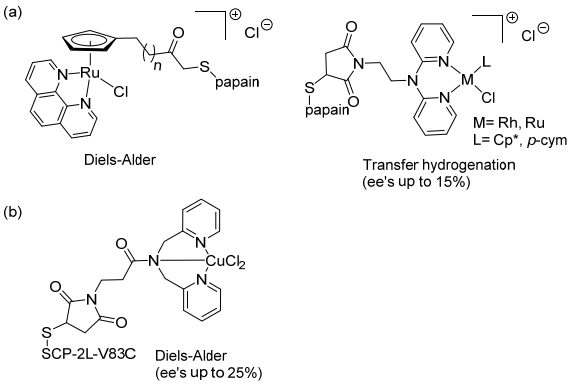
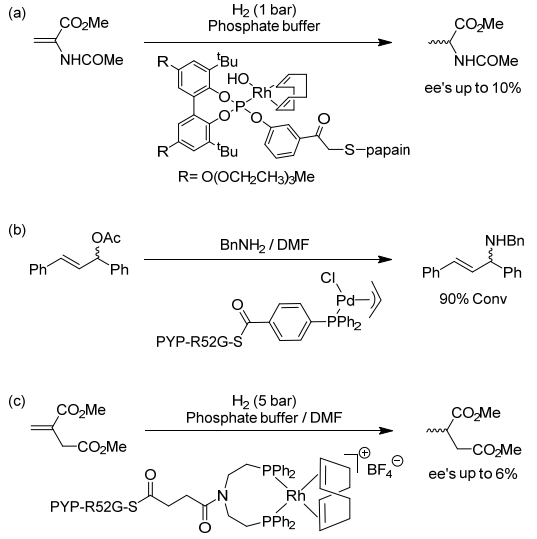


Figure 1. Examples of complexes containing nitrogen-donor ligands that have also been covalently bind to cysteine residues.

Due to the prominent role of phosphorus-based ligands in asymmetric catalysis, cysteine bioconjugation of substituted transition metal complexes containing P-ligands has received

significant attention. De Vries and coworkers covalently linked a bulky phosphite with a pendant α-haloacyl moiety to the Cys of papain that was subsequently metalated with $[Rh(cod)_2]BF_4$.[41] The resulting Rh-metalloenzyme catalyzed the asymmetric hydrogenation of methyl 2-acetamidoacrylate, albeit with low ee's (up to 10%; Scheme 14a).



Scheme 14. Representative examples of the application of artificial metalloenzymes containing transition metal complexes modified with P-ligands.

More recently, Kamer and coworkers used the unique Cys residue of photoactive yellow protein (PYP) to covalently link phosphine moieties. As a bioconjugation method they used phosphino-imidazolides (Figure 2) to form the corresponding thioester.[42] The phosphine-substituted PYP was metalated using $[PdCl(\eta^3\text{-}C_3H_5)]$, $[Rh(cod)(CH_3CN)_2]BF_4$ and $[Rh(acac)(CO)_2]$. The resulting artificial metalloenzymes were active in the Pd-catalyzed allylic amination of *rac*-1,3-diphenyl-3-acetoxyprop-1-ene[42a] (Scheme 14b) and in the Rh-catalyzed hydrogenation of dimethyl itaconate[42b] (Scheme 14c), but no or very low enantioselectivity were reported. However, the authors found that the phosphine conjugation method of phosphino-imidazolides lacked the desired chemoselectivity when applied to other enzymes, like SCP-2L and AppA.

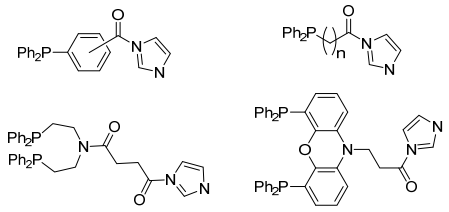
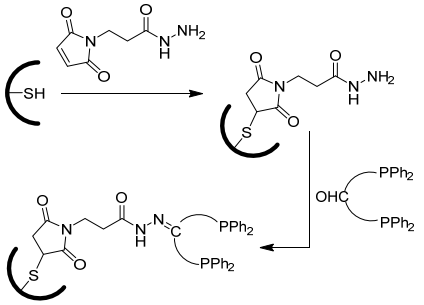


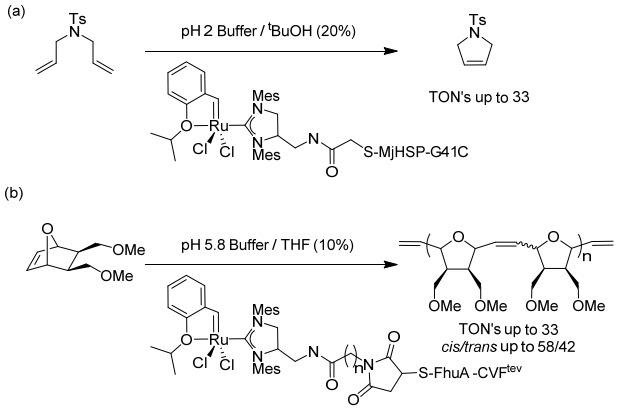
Figure 2. Phosphino-imidazolides developed to covalently bind to cysteine residues

To overcome this drawback, the same group reported a highly efficient and selective method for cysteine bioconjugation of phosphine ligands. They took advantage of the extremely high chemoselectivity of the reaction of maleimides with cysteine residues. Phosphine-containing maleimides cannot be prepared because of the nucleophilic character of the phosphine group, which leads to the formation of phosphonium salts and phosphorus ylides.[43] Therefore, they developed a highly versatile two step procedure (Scheme 15).[44] This procedure involves the initial bioconjugation of cysteine with commercially available hydrazide-substituted maleimide followed by hydrazone formation between the hydrazide and the aldehyde-substituted phosphine ligands. Upon coordination to $[Rh(acac)(CO)_2]$ a range of rhodium metalloenzymes using several enzymes and phosphines were prepared, but no application was reported.



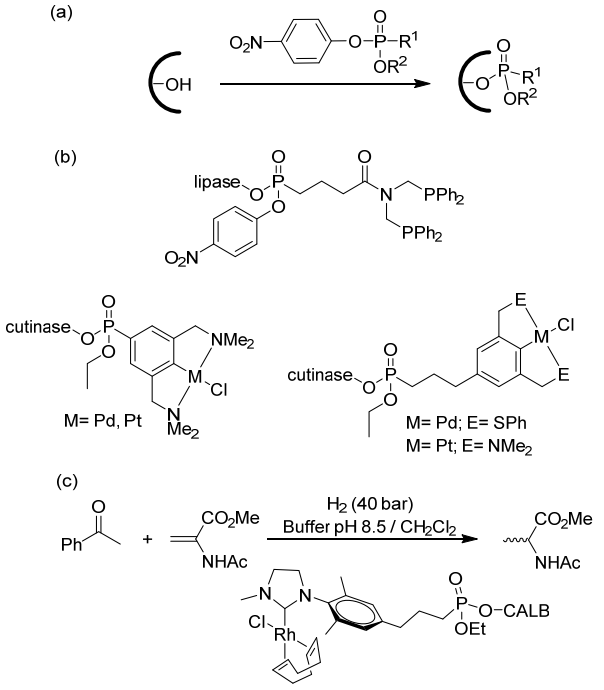
Scheme 15. General method for the bioconjugation of diphosphines in cysteine residues.

In 2011, Hilvert and coworkers demonstrated that metal-carbene complexes can also be incorporated into an enzyme.[45] Thus, a Ru-carbene olefin metathesis catalyst, modified with a bromoacetamide group, was bioconjugated in a cysteine-containing variant of a small heat shock protein (MjHSP-G41C) from *Methanocaldococcus jannaschii*. The resulting metalloenzyme was found to be active under acidic conditions in a benchmark ring closing metathesis reactions (Scheme 16a). More recently, Okuda and coworkers incorporated the same type of Ru-carbene catalyst, but modified with a maleimide moiety instead of a bromoacetamide group, in the transmembrane β-barrel protein ($FhuA\ \Delta CVF^{tev}$) extracted from *E. Coli*.[46] The resulting hybrid catalyst was successfully used in the ring opening metathesis polymerization of (1*R*,4*S*,5*S*,6*R*)-5,6-bis-(methoxymethyl)-7-oxabicyclo[2.2.1]hept-2-ene with good activities (TON's up to 990) and *cis*/*trans* selectivities up to 58/42 (Scheme 16b).



Scheme 16. (a) RCM using artificial MjHSP-G41C-Ru-carbene enzyme. (b) ROMP using hybrid FhuA ΔCVF^{tev}-Ru-carbene catalyst.

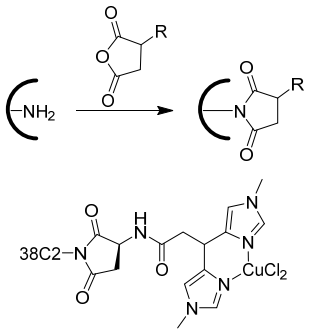
The groups of Reetz[47] and van Koten[48] early demonstrated that metal complexes can also be covalently linked to other amino acid residues other than cysteine. They took advantage of the selective bioconjugation of serine towards *para*-nitrophenyl phosphonates (Scheme 17a). A range of Rh-diphosphine and Pd- and Pt-E,C,E-pincer complexes have been linked to various serine hydrolases (Scheme 17b); however, no catalysis was reported. More recently and following the same bioconjugation strategy, Klein Gebbink and coworkers incorporated a Rh(NHC) phosphonate complex into lipases cutinase and *Candida antarctica* lipase B (CALB) (Scheme 17c).[49]



Scheme 17. Examples of hybrid metalloenzymes in which the metal complex is covalently linked to serine.

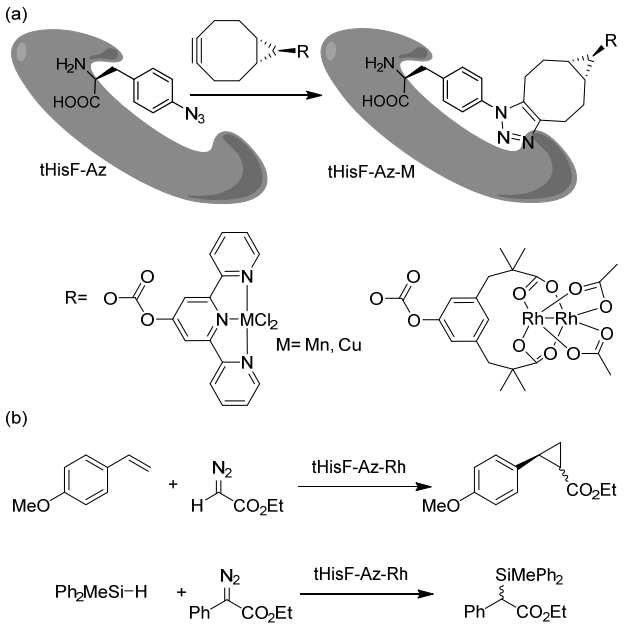
The new hybrid catalysts demonstrated an enhanced chemoselectivity in the hydrogenation of methyl 2-acetamidoacrylate over acetophenone, albeit no asymmetric induction was achieved. In particular, the use of metalloenzyme CALB-Rh(NHC), with a more sterically demanding active site than the cutinase counterpart, resulted in the exclusive hydrogenation of the olefin, leaving the ketone unreactive.

Nicholas and coworkers have shown that anhydrides can selectively bind covalently to lysine.[50] They used this bioconjugation to build artificial copper-metalloenzyme in which a copper-bis-imidazolyl complex containing a pendant anhydride is covalently linked to a lysine residue of aldolase antibody 38C2 (Scheme 18).



Scheme 18. Example of lysine bioconjugation for the preparation of metalloenzymes.

More recently a new general method for the preparation of artificial metalloenzymes has been demonstrated by Lewis and coworkers.[51] Their approach involves the incorporation of the unnatural amino acid *p*-azido-L-phenylalanine (Az) residue in the protein scaffold. The Az residue enables biorthogonal click reactions of catalytically active bicyclononyne-substituted metal complexes via strain-promoted azide-alkyne cycloaddition (Scheme 19a).[51] They therefore incorporated the Az residue in several positions of the thermostable enzyme tHisF and linked to these Az residues to Rh(II), Cu(II) and Mn(II) complexes, providing a range of artificial metalloenzymes (Scheme 19a). The tHisF-Az-Rh metalloenzymes proved to be active toward a number of carbene insertion reactions, albeit with a negligible enantioselectivity (Scheme 19b).

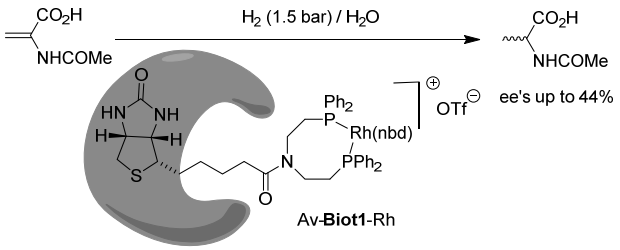


Scheme 19. (a) Preparation of novel Mn(II), Cu(II) and Rh(II)-artificial metalloenzymes via strain-promoted azide-alkyne cycloaddition. (b) Cyclopropanation and Si-H insertion using tHisF-Az-Rh hydrid catalyst.

3.3 Artifical metalloenzymes via non-covalent/supramolecular anchoring

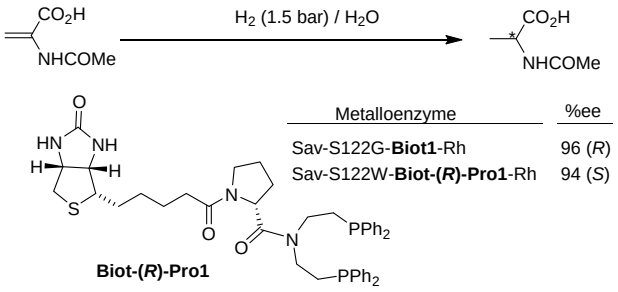
In this approach the catalytically active transition metal complex is incorporated in the enzyme using strong noncovalent highly specific protein–substrate interactions. The main advantage of this approach over the covalent anchoring is that the synthesis of the metal catalysts containing the adequate pendant anchor is easier, because reactive functional groups are not required. On the other hand, the success of this strategy relies on the strength and selectivity of the supramolecular interactions, which limits the range of enzymes that can be used. Strong supramolecular interactions are therefore necessary to guarantee quantitative, specific and steady localization of the transition-metal catalysts in the enzyme host.

The supramolecular anchoring using the biotin/(strept)avidin technology is the most studied and reliable strategy to generate metalloenzymes via non-covalent interactions. The strong biotin/(strept)avidin affinity (K_a = 10^{12} -10^{15} M^{-1}) facilitates fast and quantitative metalloenzyme formation. In the late 70's, Whitesides and coworkers disclosed the first example of the preparation of a metalloenzyme by irreversible incorporation of biotinylated Rh-diphosphine catalyst precursor to avidin (Scheme 20).[5] The new metalloenzyme Av-**Biot1**-Rh was applied to the asymmetric hydrogenation 2-acetamidoacrylic acid, affording full conversion to the product with moderate enantioselectivity (ee's up to 41% (*S*)). In the late 90's, Chan and coworkers studied whether it was possible to find a match of chirality between the chiral Rh-diphosphine catalyst and the avidin cavity with the aim to improve enantioselectivity.[52] For this purpose, they biotinylated both *R*- and *S*-forms of Pyrphos-Rh(I) complexes (Pyrphos= 3,4-bis(diphenylphosphanyl)-1-pyrrolidine) and linked them to avidin. They found that the enantioselectivity is significantly influenced by the tertiary conformation within the avidin cavity, and therefore the enantioselectivity could only be improved to 48% ee.



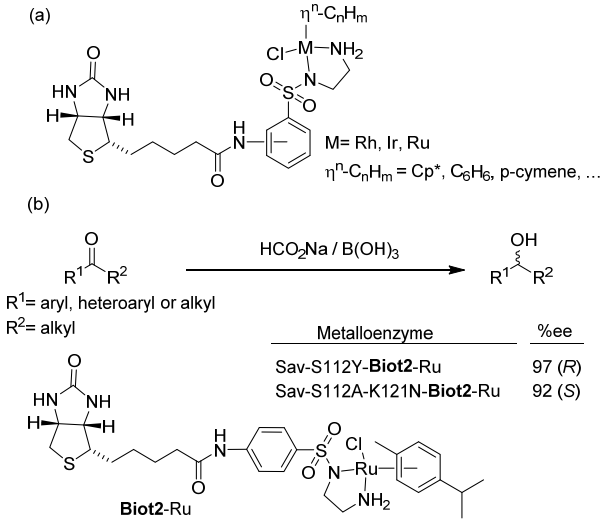
Scheme 20. Asymmetric hydrogenation of *N*-acetamidoacrylic acid using hybrid catalyst Av-**Biot1**-Rh.

An important breakthrough in this technology came in 2003 with the work of Ward and coworkers. They found that replacing the avidin by streptavidin improved the catalytic system dramatically (from 41% (*S*) ee to 92% (*R*) ee).[53] The superior performance of the streptavidin host was explained by its deeper binding pocket and its less cationic character. Ward and coworkers early made use of combined synthetic and biochemical techniques for catalyst optimization. They have therefore studied the effects in catalytic performance of: the linker, the diphosphine backbone, the introduction of several chiral elements in the ligand backbone, as well as the effect of a selected set of mutations in the streptavidin.[53,54] They were able to improve the enantioselectivity up to 96% ee (*R*) by using mutant Sav-S122G-**Biot1**-Rh metalloenzyme (Scheme 21).[53] Interestingly, the introduction of chiral amino acid spacers between the biotin anchor and the diphosphine, combined with saturation mutagenesis at position S122 of Sav afforded a second generation of Sav-Rh metalloenzymes that displays high S-selectivity (ee's up to 94% (*S*) using Sav-S122W-**Biot**-(*R*)-**Pro1**-Rh metalloenzyme) together with higher reaction rates and an improved organic solvent tolerance (Scheme 21).[54c] It should be mentioned that Reetz and coworkers took Whiteside's catalytic system and used it for directed evolution techniques for tuning the enantioselectivity of the system (as a proof-of-principle).[55] By using directed evolution they were able to increase the enantioselectivity of the methyl ester of 2-acetamidoacrylic acid from 23% ee up to 65% ee; they also inverted the enantioselectivity of the hybrid catalyst.



Scheme 21. Asymmetric hydrogenation of *N*-acetamidoacrylic acid using hybrid catalysts based on Streptavidin (Sav).

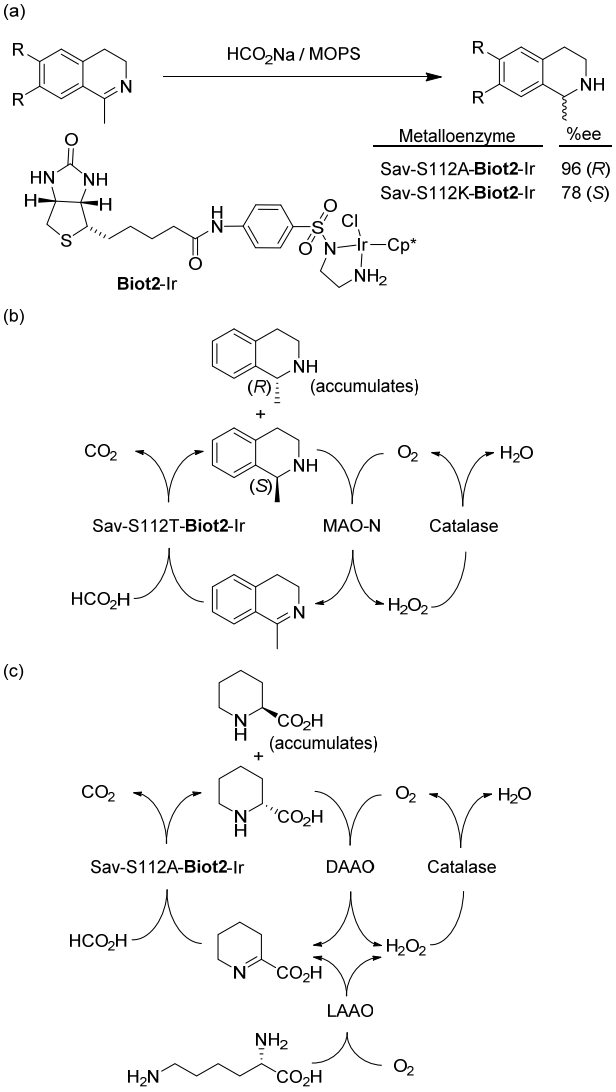
Over the past few years, Ward and coworkers have expanded their findings to cover other metal-catalyzed reactions. The next successful application involved the asymmetric transfer hydrogenation of ketones. For this purpose a range of biotinylated aminosulfonamide half sandwich complexes (Rh, Ir and Ru) were used in combination with Sav (Scheme 22a).[56] It was found that the Sav-Ru metalloenzymes are superior in terms of activity and enantioselectivity compared to the Rh and Ir counterparts and that the nature of the capping arene (either p-cymene or benzene) plays a critical role in determining which enantiomer of the product that is predominantly formed.[56b]



Scheme 22. (a) Biotinylated half sandwich complexes incorporated into streptavidin (Sav) mutants. (b) Asymmetric transfer hydrogenation of ketones using hybrid catalysts based on Sav.

The catalyst design was further optimized based on information from the X-ray structure of Sav-S122K-**Biot2**-Ru that indicated that positions Lys-121 and Leu-124 may interact with the arene and the incoming substrate.[56c] As a result, both enantiomers of the alcohol products can be obtained from aromatic and aliphatic ketones in high enantioselectivity (ee's up to 97%), provided that the difference in steric bulk of the substituents on the ketone is large enough (Scheme 22b).[56]

Recently, the Ward group focused their attention on the asymmetric transfer hydrogenation of cyclic imines. In the first experiments, they used the already developed hybrid catalyst for the transfer hydrogenation of ketones.[57] In contrast to the reduction of ketones, Sav-Ir hybrid catalysts proved to be superior to the Ru and Rh analogues in the reduction of imines. They found that both enantiomers of the reduced amine could be achieved in 96% ee (*R*) and 78% ee (*S*) upon substitution of Ser112 to either alanine (S112A) or lysine (S112K), respectively (Scheme 23a).

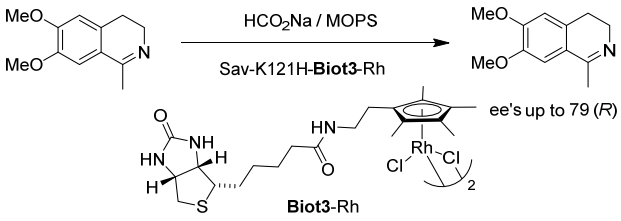


Scheme 23. (a) Asymmetric transfer hydrogenation of cyclic imines using hybrid Sav-**Biot2**-Ir catalysts. (b) Enzyme cascade for the double stereoselective deracemization of amines. (c) Enzyme cascade for the production of L-pipecolic acid from L-lysine.

Combining both docking and saturation kinetic studies indicated that enantioselection follows an "induced lock-and-key mechanism", i.e. Sav dictates the configuration of the biotinylated Ir-complex

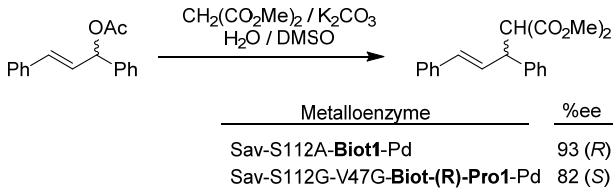
which, in turn, by and large determines the enantioselectivity of the metalloenzyme. Interestingly, the same authors have recently shown that Sav-**Biot2**-Ir hybrid catalysts are fully compatible with and complementary to a variety of natural enzymes, thus leading to the development of concurrent redox synthetic cascades.[58] Thus, for instance, Sav-**Biot2**-Ir hybrid catalysts were combined with enzymes MAO-N (monoamine oxidase from A. niger) and a catalase (from bovine liver) to afford a double stereoselective deracemization of amines (Scheme 23b). Another example is the production of L-pipecolic acid from L-lysine (Scheme 23c) that involves up to four enzymes: L-amino acid oxidase from C. atrox (LAAO), Sav-**Biot2**-Ir, D-amino acid oxidase from porcine kidney (DAAO) and catalase (from bovine liver).

More recently, they developed biotinylated Rh- and Ir-half sandwich complexes in which the biotin anchor is linked to the cyclopentadienyl moiety (Scheme 24).[59] This strategy leaves three coordination sites available for catalysis and/or activation via additional ligands. DFT studies together with mutagenesis experiments indicated that the introduction of a suitable positioned histidine residue contributes to firmly anchor this biotinylated piano stool complexes via dative bonds. This dual anchoring strategy not only helps in the positioning of the complex but at the same has a significant positive effect on both activity and selectivity. Thus, the Sav-K121H-**Biot3**-Rh catalyst quantitavely reduced 6,7-dimethoxy-1-methyl-3,4-dihydroisoquinoline with 79% ee (Scheme 24).



Scheme 24. Asymmetric transfer hydrogenation of cyclic imines using Sav-**Biot3**-Rh hybrid catalysts.

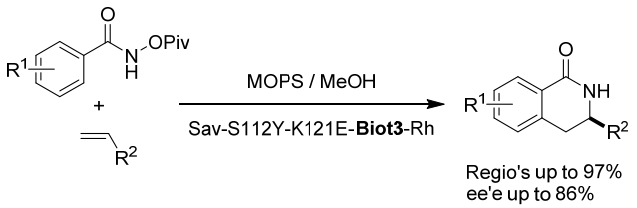
In 2008, Ward and coworkers evaluated the biotinylated diphosphine ligands, previously used in the asymmetric hydrogenation, in the asymmetric Pd-catalyzed allylic alkylation of *rac*-1,3-diphenyl-3-acetoxyprop-1-ene, which has no equivalent in enzymatic catalysis.[60] As observed in the asymmetric hydrogenation, by suitable choosing the linkers and the mutations both enantiomers of the alkylated product were achieved with enantioselectivities up to 93% ee (Scheme 25).



Scheme 25. Asymmetric allylic alkylation of *rac*-1,3-diphenyl-3-acetoxyprop-1-ene using hybrid catalysts.

Ward and coworkers also incorporated biotinylated Mn-salen complexes into several mutants of Sav. However, only moderate conversions and low enantioselectivities were achieved in the sulfoxidation of thioanisole.[61]

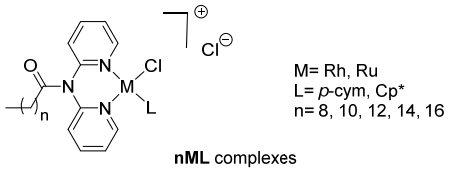
More recently, Ward and coworkers took advantage of the general design that can be used in a variety of catalytic reactions of previously reported biotinylated piano stool Rh(III) complexes **Biot3**-Rh (Scheme 24) to study the Rh-catalyzed asymmetric C-H activation.[62] Preliminary results using WT-Sav-**Biot3**-Rh catalyst provided only traces of product after 36 h. With the aim of increasing catalytic activity the authors decided to introduce a basic residue close to the Rh-center (positions S112 and $K121_B$ of the adjacent Sav monomer B). A carboxylate residue at position 121 was found to be essential for achieving an efficient asymmetric C-H activation via a concerted cyclometalation/deprotonation mechanism. The coupling of several benzamides and alkenes therefore proceeded with high activities (up to 100-fold rate acceleration compared to the isolated Rh-complex) and enantioselectivities (up to 86% ee) using Sav-S112Y-K121E-**Biot3**-Rh catalyst (Scheme 26).



Scheme 26. Asymmetric C-H activation using biotinylated Rh(III)-complex in Sav.

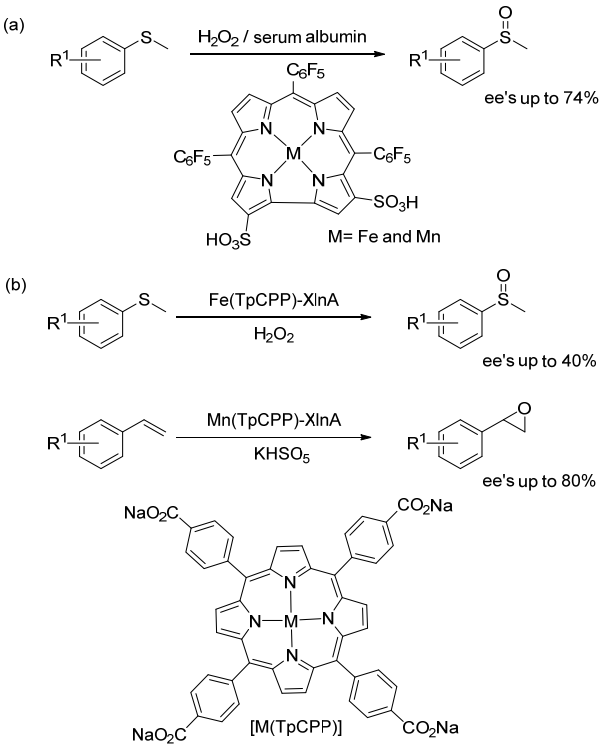
Recently, the Ward group has also biotinylated a Ru-Grubbs type metathesis catalysts and incorporated it in both avidin and streptavidin.[63] The new Ru-metalloenzymes are active in the ring-closing metathesis of *N*-tosyldiallylamine in aqueous and organic solvents. The results indicated that the position of the biotin-moiety on the carbene ligand was found to critically influence the catalytic activity. It is expected that further modifications via site-directed mutagenesis will help to further improve the catalytic performance as well as to tackle stereoselectivity issues.

Another important supramolecular approach for the generation of metalloenzymes relies on the presence of a large hydrophobic cavity in the enzyme to which hydrophobic guests, such as binaphthyl moieties[64] or fatty acid residues[65], can be incorporated. In this respect Salmain and coworkers chose bovine β-lactoglobulin (β-LG) to prepare new Ru and Rh-metalloenzymes for transfer hydrogenation of ketones.[65] The β-LG hydrophobic cavity allows the binding of a range of fatty acids with submicromolar dissociation constants.[66] The fatty acid aliphatic chain occupies the cavity with the carboxylic group directed towards the cavity entrance. A series of half sandwich Ru- and Rh-complexes containing dipyridyl ligands with saturated fatty acids (**nML** complexes) were linked into the β-LG secondary sphere (Scheme 27). The new metalloenzymes catalyzed the transfer hydrogenation of activated aryl ketones in water with formate as the hydrogen source with low-to-moderate enantioselectivities (ee's up to 32% using β-LG-**16RhCp*** metalloenzyme).



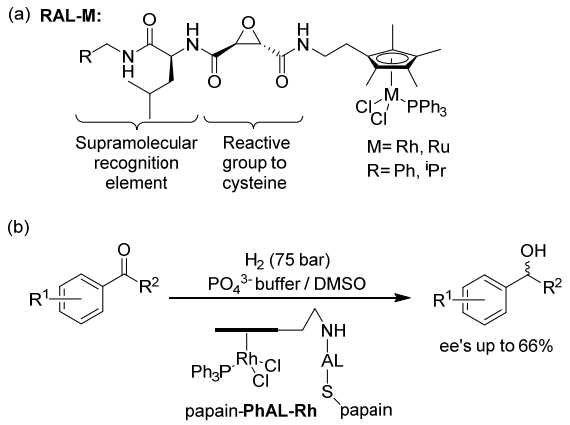
Scheme 27. Half sandwich complexes **nML**, containing fatty acids, incorporated into bovine β-lactoglobulin (β-LG).

Another non-covalent interaction that has been used for the preparation of artificial metalloenzymes makes use of electrostatic interactions. Gross and coworkers have taken advantage of the high affinity of serum albumins for anionic porphyrins.[67] Thus, a range of new artificial metalloenzymes were prepared by incorporating a bis sulfonated Fe or Mn corrole to a range of serum albumines such as those from human, bovine, porcine, and rabbit (Scheme 28a). The new hybrid catalysts catalyzed the sulfoxidation of thioanisole derivatives with ee's up to 74%. The albumine-conjugated Mn-corrole not only provided higher activity and enantioselectivity than that of the Fe-counterparts but also displayed catalase-like activity, being oxidant-coordinated Mn(III) species key intermediate.[68] More recently, Mahy and coworkers have also taken advantage of the positively charged character of the catalytic site of xylanase A (XlnA) from *Streptomyces lividans* to incorporate anionic phorphyrins such as Fe(T*p*CPP) and Mn(T*p*CPP) (TpCC=*meso*-tetrakis(*p*-carboxyphenyl)phorphyrin).[69] Fe(T*p*CPP)-XlnA was able to catalyse the sulfoxidation of thioanisole using H_2O_2 with ee's up to 40% in the presence of imidazole as cocatalysts (Scheme 28b).[69a] On the other hand, Mn(T*p*CPP)-XlnA successfully catalyzed the epoxidation of a range of styrenes by $KHSO_5$ (ee's up to 80%; Scheme 28b).[69b]



Scheme 28. (a) Asymmetric sulfoxidation using hybrid metalloenzymes containing M-corrole units. (b) Asymmetric sulfoxidation and epoxidation using [M(TpCPP]-XlnA hybrid catalysts.

As mentioned above the non-covalent/supramolecular approach requires highly specific protein–substrate interactions (e.g. biotin-(strept)avidin). This fact limits the binding of metal complexes to other enzymes. To address this drawback Eppinger and coworkers have recently introduced a new concept for the preparation of artificial metalloenzymes. This new strategy uses specifically designed metal-conjugated affinity labels to introduce transitions metal complexes inside the binding pockets of enzymes.[70] The design of the affinity labels (AL), which is similar to biogenic cofactors, combines a supramolecular recognition element to direct the binding orientation together with a reactive group that will form a covalent bond. To demonstrate this concept the authors bound half sandwich metal species containing conjugated affinity labels (**RAL-M**) to calpain inhibitor E64c (Scheme 29). Calpain inhibitor E64c efficiently inhibits cysteine proteases (such as papain) through S-alkylation of the reactive epoxide of the epoxysuccinyl ester moiety by the active site cysteine. The metal conjugated affinity labels were successfully incorporated to bromelain and papain as protein hosts. The new metalloenzymes were active in the asymmetric hydrogenation of ketones. Ligand acceleration aided to translate the chirality of the enzyme host to the product outcome (ee's up to 66% using papin-**PhAL-Rh** hybrid catalyst; Scheme 29b).

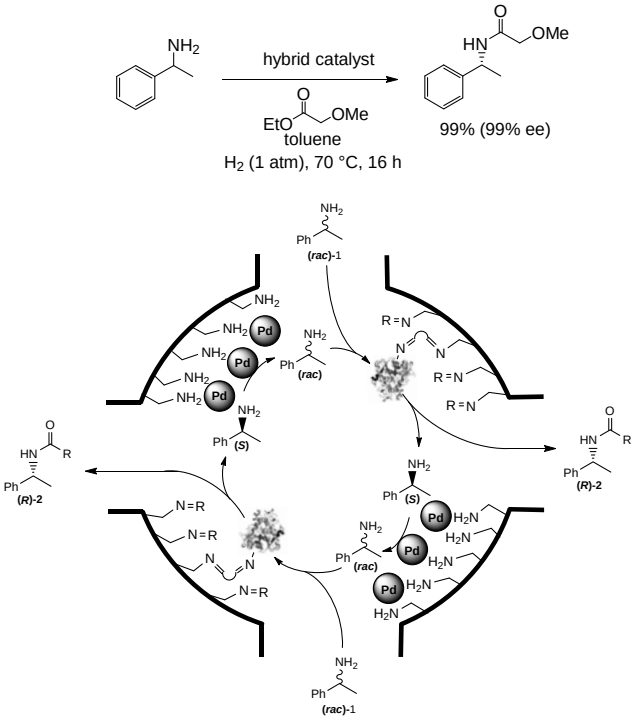


Scheme 29. (a) Half-sandwich metal conjugated affinity labels (**RAL-M**), inspired from calpain inhibitor E64c, developed for specific binding to cysteine proteases. (b) Asymmetric hydrogenation of ketones using hybrid catalyst papain-**PhAL-Rh**.

4 Hybrid catalysts with metalloenzyme-like properties

A limitation with the approach of binding a metal catalyst into the active site of the enzyme is that the enzyme activity is inhibited. The artificial metalloenzymes discussed above therefore do not utilize the natural activity of the enzyme to carry out a specific organic transformation (which often is enantioselective). On the other hand they take advantage of the chiral shape of the now non-active enzyme to accomplish an enantioselective transformation.

In metalloenzymatic dynamic kinetic resolution (DKR) of alcohols and amines, the enzyme, often a lipase, is carrying out an enantioselective acylation of the alcohol (amine) and the metal is continuously racemizing the non-acylated substrate. In this DKR reaction it would be challenging to create an artificial metalloenzyme that can carry out the transformation as a hybrid catalyst. In this approach one would need to maintain the activity and stereoselectivity of the enzyme with the original function of the active site. A hybrid catalyst that has the properties of a metalloenzyme was designed by Bäckvall and coworkers.[71] They co-immobilized Pd nanoparticles and the lipase *Candida antarctica* lipase B (CALB) into the cavities of a so-called mesocellular foam (MCF). Aminopropyl-functionalized MCF was allowed to react with Pd(II) (as Li_2PdCl_4), which strongly binds to the amino groups. The Pd(II) was subsequently reduced (by $NaBH_4$) to nanoparticles (~2 nM). A number of aminopropyl groups were left free for the enzyme and these free aminopropyl groups of the support were functionalized with glutaraldehyde. The free aldehyde group of glutaraldehyde was then used as linker for binding in the lipase (CALB) via amino groups in lysine and arginine residues. In this way a hybrid catalyst with metalloenzyme-like properties was obtained, which can perform DKR of amines. DKR of 1-phenylethylamine using ethyl methoxy acetate as the acyl donor under 1 atm. of H_2 at 70 °C afforded the desired (*R*)-amide product in 99% yield and 99% ee within 16 h (Scheme 30).



Scheme 30. DKR of 1-phenylethylamine using hybrid catalyst with metalloenzyme-like properties

Filice *et al.* synthesized a protein-inorganic hybrid by mixing CALB with $Pd(OAc)_2$ in aqueous media.[72] The palladium(II) was reduced by the enzyme to give a hybrid catalyst with metalloenzyme-like properties that catalyzed a DKR of 1-phenylethylamine with ethyl acetate at 70°C in 98% yield and 99% ee.

Another example of a hybrid catalyst with metalloenzyme-like properties was described by Kim and coworkers.[73] Pt nanoparticles were introduced into an aminopeptidase from *Streptococcus pneumonia* (PepA) by slowly reducing K_2PtCl_4 by $NaBH_4$ in the presence of the enzyme. The hybrid catalyst formed kept the natural catalytic activity of the enzyme and the metal activity of the Pt nanoparticles; it was used in a two-step cascade reaction, involving enzyme-catalyzed amide bond cleavage and Pt-catalyzed hydrogenation.

5 Entry points to the preparation and use of artificial metalloenzymes

To the non-specialist classical organic and inorganic chemists the preparation and use of hybrid metalloenzymes can seem a daunting task hindered by the complexity of the enzymes compared to classical chemical catalysts. However, the structure of many enzymes is known and over the past years there have been great advances in enzyme computer

modeling.[74] These features not only facilitate the selection of suitable amino acid residue for bioconjugation but also allow visualizing how the structure of the enzyme may influence both the activity and the stereochemical outcome of the catalytic reaction. Therefore, knowledge about the nature of the position of the different residues close to the non-native metallic catalysts is crucial not only to prevent deactivation of the metal center due to interactions with other amino acid residues but also, and more importantly, to protect the organometallic catalysts towards external factors that may cause deactivation.[75] In addition, computer modeling should facilitate the metalloenzyme design with the aim of maximizing the enantioselectivity for a given catalytic reaction.

The selection of the appropriate bioconjugation method for site selective anchoring of different metal residues to proteins is another important issue that a chemist should face in the design and synthesis of hybrid metalloenzymes.[76] However, this is facilitated by the large plethora of bioconjugation strategies developed over the past two decades.[77]

Most of the examples on the use of artificial metalloenzymes in asymmetric catalysis reported up to date make use of water as solvent.[78] However, the high reactivity of water and the low solubility of the vast majority of organic molecules hamper the extension of artificial metalloenzymes to the large diverse set of asymmetric catalytic transformations catalyzed by transition metals. There are however a large number of enzymes that maintain their activity in organic solvents.[79] The use of this latter class of enzymes may facilitate the development of metalloenzymes for a diverse class of metal-catalyzed asymmetric transformations.

Another factor that should stimulate chemist to explore this new frontier is the commercially availability of many enzymes, so there is no need to spend time and economic resources in the development of laboratories for the production and purification of enzymes. Although, it is advisable to gain this kind of expertise to speed up the development of mutated enzymes or to introduce a specific binding site as examples.

6 Future perspectives and conclusions

Over the past two decades, the field of artificial metalloenzymes has been continuously growing and many important groups are searching for new hybrid catalysts that are more active, selective and environmentally friendly. The biotin-(strept)avidin strategy has emerged as one of the most successful approaches, and it has therefore been effectively applied in several asymmetric transformations. However, its application to other relevant enantioselective reactions is still missing. In comparison the successful application of metalloenzymes that use other bioconjugation approaches to anchor the metal center to the enzyme has been scarce. In most of the cases the failure in transferring the chiral information from the enzyme to the product can be attributed to either the presence of multiple metal binding sites, which lead to the formation of mixtures of catalytic species with different chiral environments within the artificial metalloenzyme, or to the incorrect choice of the enzyme. During the last couple of years, however, new highly selective bioconjugated strategies have been successfully implemented for the preparation of artificial metalloenzymes with the metal fragment exclusively bound to the desired position. These advances will be crucial for the construction of highly selective hybrid catalysts in the near future.

Regarding the choice of the enzyme, the range of enzymes used for the preparation of the hybrid catalyst has been very narrow. It is easy to envisage that in the near future the focus of the research will be on how to explore the large variety of enzymes available in the quest for the highest levels of enantioselectivity. Nevertheless, the enormous amount of enzymes available together with the great diversity of organometallic species that can be incorporated offers an endless amount of possible artificial metalloenzymes to be prepared for a given transformation, which is impossible to analyze even with the more advanced combinatorial techniques. Therefore, the implementation of a rational computational modeling, together with directed evolution and combinatorial techniques, will be pivotal in the selection of the right enzyme.

Another important aspect that researchers should focus on is to develop new strategies to match the excellent activities found in natural enzymes, which is one of the main advantages of using enzymes compared to traditional metal-catalysts. To date most of the artificial metalloenzymes display lower activities than homogeneous metal-catalysts.

In summary, by combining the concepts of biology for selective recognition with those of metal catalysis, it is possible to develop novel highly selective catalysts for asymmetric catalytic reactions. The high substrate specificity will allow conversion of a single substrate present in complex mixtures, like those in biological systems.

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REVIEW

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