Artificial metalloenzymes: proteins as hosts for enantioselective catalysis

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Enantioselective catalysis is one of the most efficient ways to synthesize high-added-value enantiomerically pure organic compounds. As the subtle details which govern enantioselection cannot be reliably predicted or computed, catalysis relies more and more on a combinatorial approach. Biocatalysis offers an attractive, and often complementary, alternative for the synthesis of enantiopure products. From a combinatorial perspective, the potential of directed evolution techniques in optimizing an enzyme's selectivity is unrivaled. In this review, attention is focused on the construction of artificial metalloenzymes for enantioselective catalytic applications. Such systems are shown to combine properties of both homogeneous and enzymatic kingdoms. This review also includes our recent research results and implications in the development of new semisynthetic metalloproteins for the enantioselective hydrogenation of *N*-protected dehydroamino acids.

1. Introduction

The preparation of enantiomerically pure compounds for the production of pharmaceuticals, agrochemicals, flavors, and functional materials is one of the most actively pursued fields in synthetic chemistry.[1,2] The possibility of extending the patent-life of a racemic compound by protecting a single enantiomer (the so-called "chiral switch") attracts much interest from industry in this area. It is interesting to note that, despite the Thalidomide tragedy, only 40% of all dosage-form drug sales in 2000 were of single enantiomer. The worldwide sales of chiral drugs in single enantiomer dosage form have been growing at an annual rate greater than 10% in the past years. It is estimated that the future growth rate will yield worldwide sales greater than 200 billion Euros by 2008.[1]

Three main routes exist for the preparation of enantiomerically pure compounds: (i) enantiomer separation; (ii) transformation of a chiral precursor from the chiral pool and (iii) enantioselective reactions, either stoichiometric or catalytic.

In terms of atom economy, the use of enantioselective catalysts, either heterogeneous, homogeneous or enzymatic, is the most efficient and attractive. Metal-catalyzed enantioselective transformations have received much attention during the past three decades and have played a crucial role in the development of modern synthetic methodologies.[3] This

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Christophe Thomas was born in Paris in 1972. He studied chemistry at Strasbourg University and moved to the University of Neuchâtel in 1998. He obtained his PhD degree in 2002 working on the rhodium-catalyzed carbonylation of methanol under the supervision of Professor Süss-Fink. He then joined Professor Coates' group at Cornell University as a postdoctoral fellow supported by the Swiss National Science Foundation (2002–2003) and worked on the development of cobalt-based complexes for the copolymerization of carbon dioxide and epoxides. After spending one year as a postdoctoral fellow in Professor Ward's laboratories in Neuchâtel, he was appointed Assistant Professor at the University of Rennes in 2004. His research interests comprise the study of fundamental processes in organometallic chemistry, the application of organometallic complexes as catalysts for homogeneous catalysis.

Thomas Ward

Thomas Ward was born in Fribourg in 1964. Following his diploma (1987, University of Fribourg), he joined Professor Venanzi at the ETH Zürich. His PhD dealt with the synthesis and catalytic properties of C_3-symmetric phosphine ligands. In 1991 he joined the group of Professor R. Hoffmann (Cornell University) as postdoctoral fellow. Following a short stay in the group of Professor C. Floriani (University of Lausanne), he was awarded an A. Werner stipend and moved to the University of Berne to undertake his independent career in Fall 1993. In Fall 2000 he was appointed Professor at the University of Neuchâtel. His research interests focus on the exploitation of proteins as second coordination sphere ligands in catalysis and in nano-biotechnology.

endeavor was rewarded with the 2001 Nobel Prize in Chemistry for achievements in this field.[4–6]

Despite these efforts, it remains very difficult to predict the outcome of a metal-catalyzed enantioselective reaction. Indeed, the differences in energy involved in the transition states leading to both enantiomers of the desired product are too small to be reliably predicted. As a consequence, the number of efficient enantioselective metal catalysts and the corresponding substrates remains modest.[7]

In parallel to these developments, enzymatic catalysis has emerged as an alternative tool for the synthesis of enantiopure compounds.[8,9] Both the academic- and the industrial community view biocatalysis as a highly promising area of research, especially for the development of sustainable technologies for the production of complex active ingredients in pharmaceuticals and in agrochemicals. Table 1 outlines some of the most noteworthy features of homogeneous- and of enzymatic catalysis.

Enzymes exquisitely tailor both the first- and the second coordination sphere of their active site to afford efficient and selective catalytic systems. This characteristic is particularly difficult to achieve for homogeneous catalysts. Indeed, the steric- and the electronic control of a homogeneous catalyst is mostly limited to the first coordination sphere of the metal. However, the role of the solvent and of the counterion (typical second coordination sphere interactions) often plays a determinant role in the performance of a catalyst.

Biocatalysts are attractive because of their remarkable chemo-, regio-, and stereoselectivity, their impressive catalytic efficiency, and their reactivity in aqueous media. Although these characteristics can be useful, they can also limit the exploitation of biocatalysts.[10]

Homogeneous catalysts are often more tolerant than enzymes towards variation in size of a substrate because of the inherent single-handed, lock-and-key specificity of biocatalysts. Despite their difference in size, acetamidoacrylic- and acetamidocinnamic acid are often both good substrates for homogeneous hydrogenation catalysts.

Organometallic- and enzymatic catalysts generally exhibit their most characteristic activities on different classes of reactants: organometallic catalysts are especially effective with nonpolar substances (*e.g.*, H_2, CO, and olefins), while enzymatic catalysts are often most useful with polar, polyfunctional materials (*e.g.*, carbohydrates, acid derivatives, and biopolymers).

Biocatalysts have evolved to target a single functionality even in the presence of other, perhaps more reactive, functionalities: enzymes do not require protective groups.

Despite the initial high promises, the implementation of biocatalytic processes so far has only been realized for a few large-scale operations.[9,10] In many cases, the industrial application of enzymes is hampered by the instability of the biocatalyst under process conditions.

Some of the inherent limitations of biocatalysts—including small substrate scope, operational stability, and availability of only one enantiomer of the product—have been recently overcome, thus significantly expanding the scope of applications of biocatalysis. This has been made possible by the implementation of new methods for evolving proteins of interest and essaying their activity. Approaches to the design of catalytic proteins include automated sequence design, in which a novel catalytic site is engineered into a natural protein by mutating a subset of residues, and *de novo* protein design, which requires the simultaneous design of the entire backbone structure and sequence.[11–15] Directed evolution (*i.e.* genetic optimization) combined with high-throughput screening have significantly facilitated the optimization of customized enantioselective enzymes.[16–20] Although genetic engineering techniques can improve the overall stability, the activity and the selectivity of a biocatalyst, the *de novo* design of enzymes remains very challenging. Monoclonal catalytic antibodies open interesting opportunities in this field however.[21,22]

From these considerations, it appears that homogeneous- and enzymatic catalysis are, in many respects, complementary. In a biomimetic spirit, efforts are currently underway in different groups to design enantioselective artificial metalloenzymes.[23] The introduction of an achiral catalytic moiety, which ensures activity, in a chiral pocket provided by a host protein is expected to produce active and (enantio)selective hybrid catalysts with a well defined second coordination sphere provided by the host protein. Such hybrid systems should display properties reminiscent of both homogeneous- and enzymatic kingdoms.

2. Preparation of artificial metalloenzymes

To ensure localization of the metal-containing moiety (the coenzyme) within the host protein, two complementary strategies are currently being pursued by various groups for the generation of artificial metalloenzymes:

(i) Covalent incorporation of a metal-containing coenzyme (Section 2.1)

Table 1 Typical features of homogeneous- and enzymatic catalysis

	Homogeneous catalysis	Enzymatic catalysis
Enantiomers	Both enantiomers accessible	Single enantiomer
Reaction repertoire	Large	Small
Turnover numbers	Small	Large
Solvent compatibility	Organic > Aqueous	Aqueous > Organic
Optimization	Chemical	Genetic
Second coordination sphere	Ill-defined	Well-defined
Functional group tolerance	Small (protective groups)	Large
Substrate scope	Large	Limited
Typical substrates	Apolar	Flexible

(ii) Non-covalent (*i.e.* supramolecular) incorporation of a metal-containing coenzyme (Section 2.2).

Both approaches rely on the incorporation of a metal-containing catalyst in a binding pocket provided by the protein. To ensure unambiguous localization of the coenzyme, the host protein must contain a binding site or a unique functionality which reacts (quasi) irreversibly with the coenzyme or a portion thereof (the anchor) (Fig. 1).

2.1. Covalent anchoring

Kaiser was the first to demonstrate that novel active sites can be introduced into enzymes by the chemical modification of specific amino acid residues with the use of appropriately designed coenzyme analogs.[24] His group described that papain can be converted into a highly effective oxido-reductase by covalent modification of the sulfhydryl group of the active site cysteine residue (Cys25) with flavins (Fig. 2). The resulting flavopapains react with dihydronicotinamides and exhibit rate enhancements (up to 670 fold) over native enzymes.

Inspired by this work, several groups have recently developed methods to covalently modify proteins by *incorporating transition-metal complexes* to yield hybrid catalytic systems with promising catalytic properties. Among these, Rana and Meares have investigated the use of ferrous-EDTA as metal chelate for the incorporation of transition metals into proteins.[25] Site-specific conjugation with either bovine serum albumin or human carbonic anhydrase I (BSA and HCA I respectively) was achieved by alkylation of the only free sulfhydryl (Cys34 and Cys212 respectively) with 1-(*p*-bromoacetamidobenzyl)-EDTA. The chelating group was loaded with an Fe(III) salt and the resulting artificial metalloenzymes were shown to perform intramolecular (*i.e.* single turnover) protein cleavage with a high regiospecificity, yielding three- and two protein fragments respectively (for BSA and HCA I conjugates).[25]

Building on earlier work on thiolsubtilisin and from the unique chemistry of selenium, Hilvert developed a methodology for the conversion of the active site serine of subtilisin

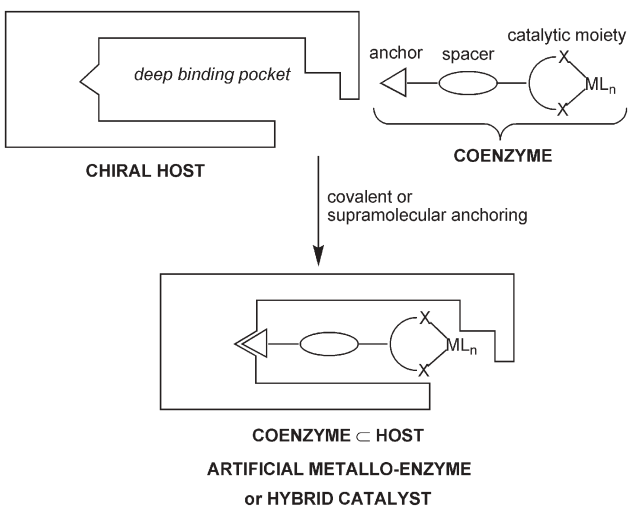

Fig. 1 Anchoring of an active catalyst within a chiral host to produce hybrid catalysts.

(Ser221) into selenocysteine.[26,27] The semisynthetic enzyme was shown (i) to function as an acyl transferase and (ii) to be able to mimic the catalytic behavior of glutathione peroxidase.

Selenosubtilisin catalyzes the reduction of alkyl hydroperoxides by thiols. The reduction of *tert*-butyl hydroperoxide (t-BuOOH) by 3-carboxy-4-nitrobenzenethiol catalyzed by selenosubtilisin occurred at least 70 000-fold faster compared to the same reaction catalyzed by diphenyl diselenide, a well-studied antioxidant. The selenosubtilisin-catalyzed reaction proceeds through a "ping-pong" mechanism involving at least one covalent intermediate as depicted in Fig. 3.

Moving towards enantioselective artificial metalloenzymes, Distefano and coworkers have reported the preparation of a 1,10-phenanthroline conjugate of an adipocyte lipid binding protein (ALBP–Phen). Iodoacetamido-1,10-phenanthroline reacts with the unique cysteine residue (Cys117) located in the interior of ALBP to afford a phenanthroline linked *via* a thioether. The semisynthetic apo-enzyme was loaded with Cu(II) and the resulting ALBP–Phen–Cu(II) was found to catalyze the enantioselective hydrolysis of several unactivated amino acid ester substrates (0.7–7.6 TON, ee 31–86%, rates 32–280 above background, Fig. 4).[28]

In the area of catalytic antibodies, several groups have covalently modified haptens to introduce a metal-containing moiety. Janda and coworkers described the preparation of a semisynthetic copper(II)-based catalytic antibody, by altering the sidechain of the active-site lysine of catalytic antibody 38C2 to an imide-linked bisimidazole sidechain.[29] The authors were able to switch an aldolase catalytic antibody into a metalloprotease-like one. The resulting 38C2-Cu conjugate is an active catalyst for the hydrolysis of a coordinating ester, following Michaelis–Menten kinetics [k_{cat} = 2.3 min^{-1} and K_m = 2.2 mM] with a rate enhancement [k_{cat}/k_{uncat}] = 2.1 × 10^5.

In the past years, Reetz has beautifully demonstrated the possibility of optimizing the selectivity of several, active but unselective, enzymes by directed-evolution methods. This approach relies on iterative cycles of random mutagenesis coupled with an efficient enantioselectivity-screening procedure. Having identified a mutant enzyme (obtained by random mutagenesis) which displays an increased selectivity over the previous generation, the gene encoding the protein is re-subjected to a cycle of random mutagenesis, in the spirit of a Darwinistic evolutionary process.[18]

By extension of this concept, Reetz proposed to extend this methodology to evolve "hybrid catalysts" containing metal coenzymes. Thus far, they were successful in introducing various ligands and complexes thereof in the binding site of a papain (Fig. 5). The nucleophilic cysteine of papain was covalently modified by reaction with maleimide-derivatized ligands (salen, dipyridine, diphosphine). Preliminary catalytic experiments show that the hybrid manganese–salen and the hybrid rhodium–dipyridine catalysts are active in epoxidation and hydrogenation. However, low enantioselectivities (<10% ee) were observed in all cases. Alternatively, phosphine ligands bearing a *para*-nitrophosphonate funtionality can be covalently linked to the serine residue in the active site of a lipase. Unfortunately, this derivatization proved to be reversible. Parallelization, miniaturization of expression,

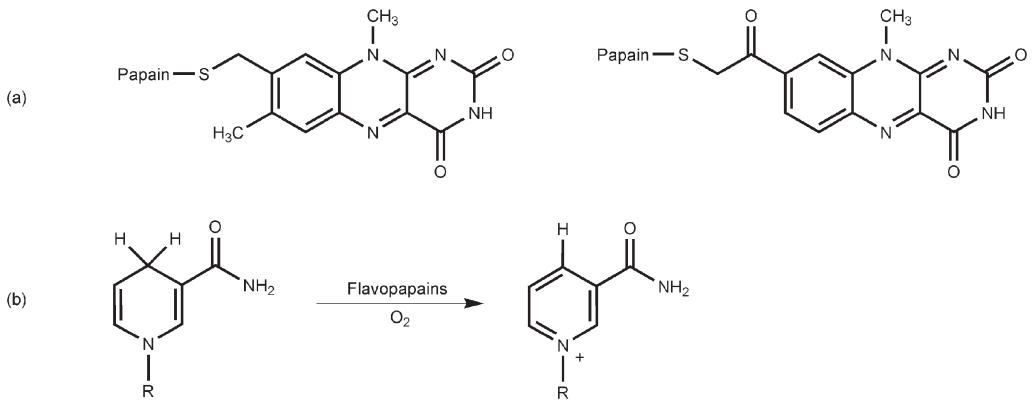

Fig. 2 (a) Examples of flavopapain derivatives synthesized by Kaiser. (b) Reaction catalyzed by flavopapains.

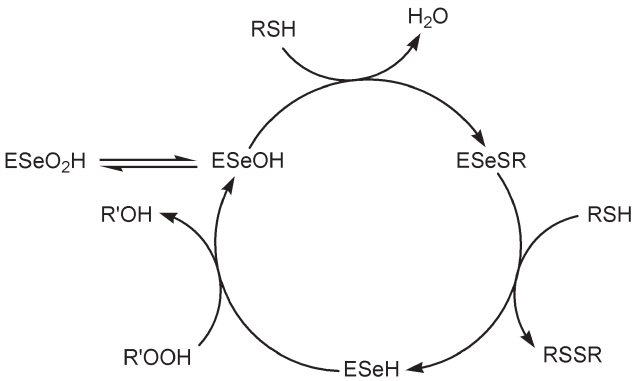

Fig. 3 Mechanism of reactions catalyzed by selenosubtilisin.

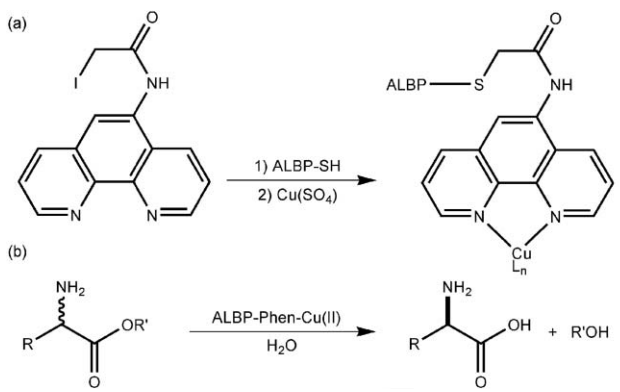

Fig. 4 (a) Preparation of ALBP–Phen–Cu(II) conjugates. (b) Enantioselective hydrolysis of amino acid ester derivatives catalyzed by ALBP–Phen–Cu(II).

protein purification, and chemical modification have yet to be accomplished.[17,18]

2.2. Supramolecular anchoring

Wilson and Whitesides were the first to convert a protein into an homogeneous enantioselective hydrogenation catalyst.[30] As early as 1978, they described an approach for the construction of an asymmetric hydrogenation catalyst based on embedding an achiral biotinylated rhodium–diphosphine moiety within avidin. Since the first coordination sphere of the catalyst is

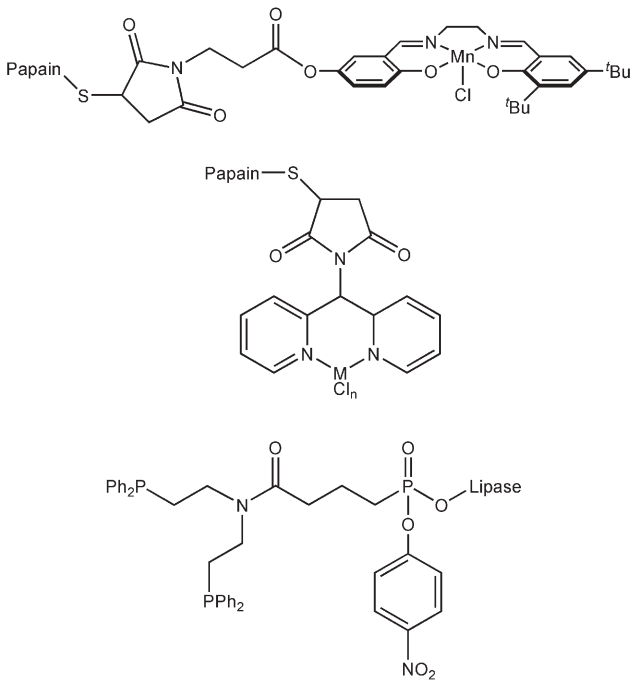

Fig. 5 Covalent attachment of Mn–salen derivatives, Cu-, Pd- and Rh dipyridine complexes and diphosphine moiety in a protein.

achiral, the protein cavity provides the chiral environment for the enantioselective hydrogenation. Avidin is a well characterized protein composed of four identical subunits, each of which binds biotin and many of its derivatives sufficiently tightly that association is effectively irreversible.[31] For the reduction of acetamidoacrylate in avidin using *N*,*N*-bis(2-diphenylphosphinoethyl)biotinamide in conjunction with Rh(I)(norbornadiene), they reported an ee = 39% in favour of the (*S*)-acetamidoalanine (Fig. 6a).

The experiments reported by Whitesides establish two principles:

(i) It is possible to carry out homogeneous hydrogenation using a $\{Rh(diphosphine)\}^+$ catalyst associated with a protein: neither the aqueous solution nor interactions between the metal and the protein deactivate the catalyst.

(ii) The chirality of the protein can induce significant levels of enantioselectivity.

Subsequently, Chan reported the use of an enantiopure biotinylated pyrphos-Rh(I) complex in conjunction with avidin for the enantioselective reduction of itaconic acid.[32] By introducing a chiral $\{Rh(I)(pyrphos)\}^+$ moiety into the protein cavity, it was found that the enantioselectivity of the system for the asymmetric hydrogenation of itaconic acid was significantly influenced by the presence of the host protein. Indeed, when combined with avidin, both (*R,R*)- and (*S,S*)-pyrphos yield the same enantiomer (*R*)-methylsuccinic acid with 11% ee and 48% ee respectively. Furthermore, they showed that the enantioselectivity critically depends on various parameters, including H_2 pressure, temperature and pH: by increasing the temperature from 22 °C to 40 °C, the absolute configuration of the product switches from 48% (*R*) to 20% (*S*) (Fig. 6b).

Using standard solid phase synthesis, Imperiali and Roy introduced a pyridoxamine cofactor, in the middle of the C-peptide derived from RNase S.[33] This synthetic tetradecapeptide which forms an alpha-helix, contains a pyridoxamine in position 8 and displays micromolar affinity for the RNase S. When loaded with Cu(II), the resulting artificial metalloenzyme exhibited a seven fold faster transamination of pyruvate to alanine for the first non-catalytic turnover of the enzyme. Upon addition of phenylalanine to recycle the catalyst however, the reaction became slower than with the free pyridoxamine, but chiral inductions up to 31% ee were obtained (Fig. 7).

More recently, Watanabe reported the insertion of achiral Cr(III) complexes into the active site of apomyoglobin (Fig. 8).[34] In myoglobin, histidine-93 binds an iron-porphyrin with a distal histidine-64 on the opposite face. Double-point mutation (His64Asp, Ala71Gly) of apomyoglobin allowed the incorporation of a $\{(Salophen)Cr(III)\}^+$ complex. Upon incorporation into the apo-myoglobin binding site, the chromium is thought to complete its coordination by binding to His93. These semisynthetic metalloenzymes were found to catalyze the enantioselective sulfoxidation of thioanisole using H_2O_2 as oxidant (up to 13% ee).

In the area of metal-containing catalytic antibodies, a few promising results have been obtained using robust porphyrin-containing haptens for immunization. The design of a metal-containing hapten is a nontrivial task because, in addition to mimicking the transition state, this hapten should have considerable hydrolytic stability under the physiological conditions during the immunization process.[35]

Following immunization, screening for binding of the antibodies, isolation and purification, the antibody is loaded with a porphyrin cofactor containing an active metal and tested in catalysis. Using this strategy, the enantioselective sulfoxidation of thioanisole was reported in two instances with promising enantioselectivities.

For immunization, Keinan and coworkers used a water-soluble tin(IV)–porphyrin hapten containing an axial α-naphtoxy ligand to mimick the transition state of the sulfoxidation of thio-anisole.[36] The catalytic assembly comprising antibody SN37.4 and a ruthenium(II) porphyrin cofactor exhibit typical Michaelis–Menten saturation kinetics. In the presence of PhIO as oxidant, thioanisole is oxidized to the corresponding sulfoxide with 43% ee in favour of the (*S*)-methylphenylsulfoxide (Fig. 9a).

Suspecting that the presence of a fifth ligand completing the coordination of the metal porphyrin is critical for activity (and is not elicited upon immunization with a metal–porphyrin hapten), Mahy and coworkers used microperoxidase 8 (MP8), a heme octapeptide where the imidazole side chain of histidine acts as a proximal ligand of the iron atom.[37] After immunization with MP8, they found that the 3A3-MP8 antibody–coenzyme combination catalyzes the epoxidation of thioanisole with 45% ee in favour of the (*R*)-enantiomer (Fig. 9b). The MP8 cofactor was found to produce essentially racemic sulfoxide in the absence of the antibody.

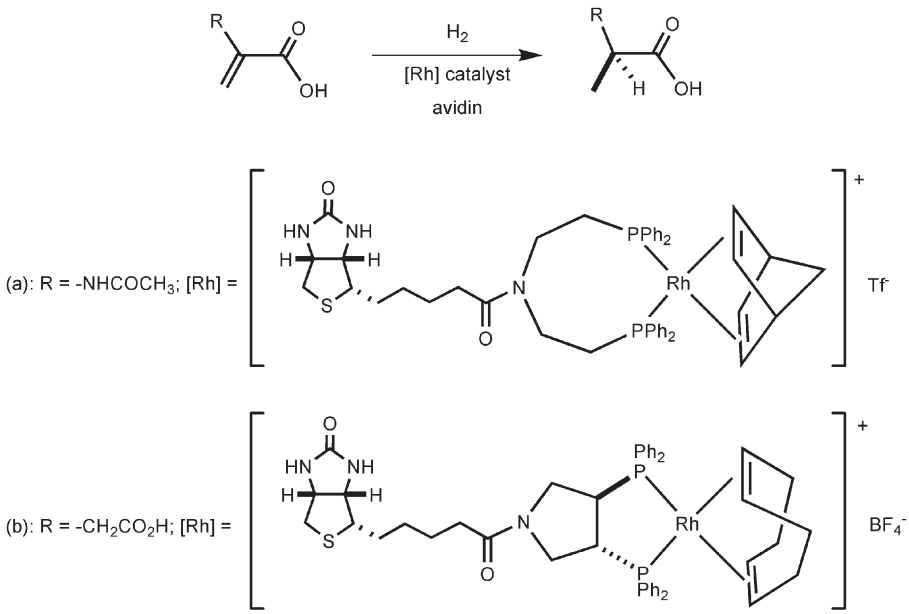

Fig. 6 (a) Enantioselective hydrogenation of acetamidoacrylic acid catalyzed by Whitesides' system. (b) Enantioselective hydrogenation of itaconic acid catalyzed by Chan's system.

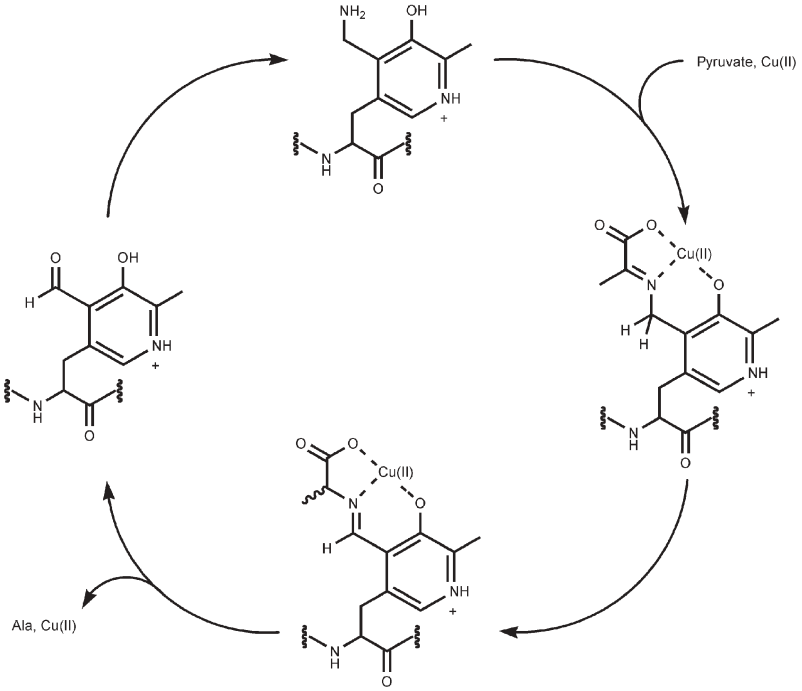

Fig. 7 Structures of intermediates in the Cu(II)-assisted transamination of pyruvate to alanine by Pam-containing peptides.

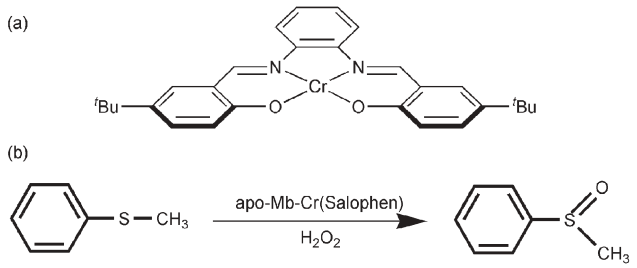

Fig. 8 (a) Salophen complex inserted into apo-Mb. (b) Sulfoxidation of thioanisole catalyzed by apo-Mb-Cr(Salophen).

Using rhodium loaded human serum albumin (HSA), Marchetti and coworkers reported the hydroformylation of styrene under biphasic conditions.[38] Although the localization of the rhodium catalyst is ill-defined (no anchoring), they report very high turnover numbers (>500 000) and good regioselectivites. For the hydroformylation of styrene, a regioselectivity is 9 : 1 in favour of the branched isomer is reported. Unfortunately, no mention of the enantioselectivity is made (Fig. 10).

Recently, Watanabe reported a strategy for the construction of a size-selective hydrogenation biocatalyst. They described the encapsulation of a Pd cluster in the apo-ferritin cavity, an iron-storage protein comprising 24 subunits that assemble to form a hollow cagelike structure of 12 nm in diameter (Fig. 11).[39] This Pd(0)-loaded-ferritin system was able to catalyze the hydrogenation of different acrylamide derivatives in water.

Based on the reports summarized above, we speculated that the most promising system to build upon is the biotin–avidin system first investigated by Whitesides. As no chemical coupling step is required upon addition of the catalyst precursor to the protein, we reasonned that the integrity of the organometallic species is warranted, thus ensuring a well defined- and localized coenzyme within the host protein. Furthermore, this system should be amenable to both chemical- and genetic optimization procedures (*i.e.* chemogenetic):

(i) The valeric acid side chain of biotin can readily be derivatized by various amino acids, which in turn may be coupled *via* an amide bond to the chelating ligand containing an amine functionality. This chemical optimization allows to probe the topography of the protein in search of a favourable chiral environment.

(ii) Avidin has been expressed in various organisms; it is a very robust protein which can easily be purified *via* affinity chromatography (on an iminobiotin resin).

As a model reaction, prototypical of homogeneous catalysis, we focused on the hydrogenation of *N*-protected dehydroamino acids (*e.g.* acetamidoacrylate). Two different biotinylated ligand scaffolds **Biot–1** and **Biot–2** were selected and tested in the presence of Rh(I) salts. In agreement with both Withesides' and Chan's observation, we found that the biotinylated ligands devoid of spacer give modest levels of enantioselection (**Biot–1** : 39% ee (*S*), **Biot–2** : 0% ee).[40]

Next, we introduced a spacer between the anchor (biotin) and the metal diphosphine moiety. The introduction of a glycine spacer $\mathbf{3^1}$ has a positive influence on the enantioselectivity to afford 57% (*S*) and 80% (*S*) using **Biot–3^1–1 and biot–3^1–2** as ligands in avidin (Fig. 12).

Streptavidin is also a biotin-binding protein isolated from *Streptomyces avidini.* Although both avidin and streptavidin display similar affinities for biotin, they possess only ~35% primary sequence homology. Inspection of both protein monomers reveals that the major structural difference is that

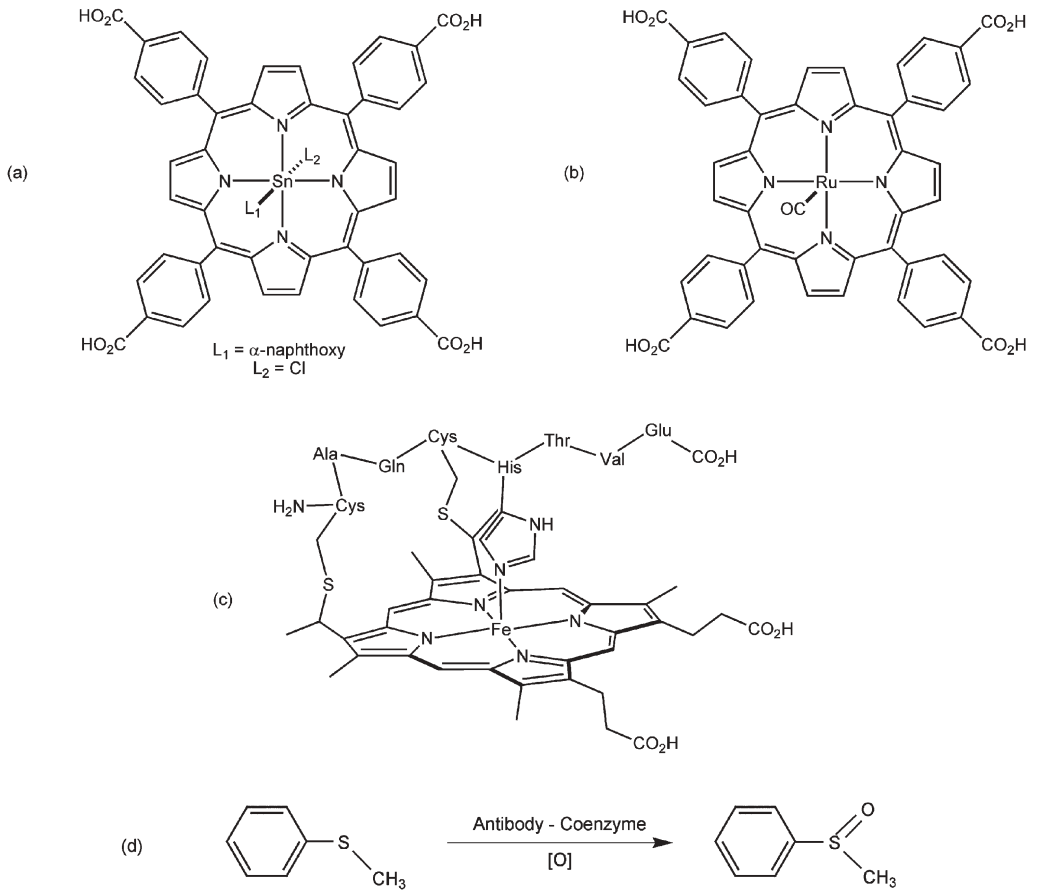

Fig. 9 (a) Hapten used by Keinan. (b) Coenzyme used by Keinan. (c) Hapten and coenzyme used by Mahy. (d) Reaction catalyzed by Keinan's and Mahy's systems.

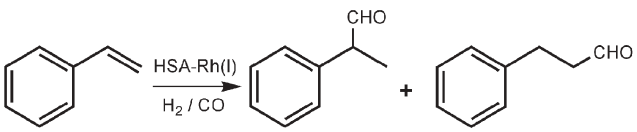

Fig. 10 Hydroformylation of styrene catalyzed by HSA-Rh(I) system.

streptavidin is endowed with a deeper binding pocket (Fig. 13).[31]

Using streptavidin as a host protein, we found that, in the presence of **Biot–1**, the reduction of acetamidoacrylate proceeds with 94% ee in favour of the (*R*)-enantiomer.

Having identified a host protein–ligand combination affording high levels of enantioselection, site-directed mutagenesis was performed on position Ser112 of wild-type streptavidin. This position was selected as molecular modelling suggests that it lies close to the rhodium center upon incorporation of the coenzyme $[Rh(COD)(\mathbf{Biot–1})]^+$ in streptavidin ($[Rh(COD)(\mathbf{Biot–1})]^+ \subset$ SAV). The streptavidin mutant Ser112Gly was thus produced in *E. coli*, purified on an iminobiotin column and screened with various ligands-spacer combinations for the reduction of acetamidoacrylate. With **Biot–1**, a slight increase in enantioselectivity was observed (96% ee (*R*), compared to 94% ee (*R*) for WT SAV). Interestingly, in the combination with **Biot–3[1]–2**, the

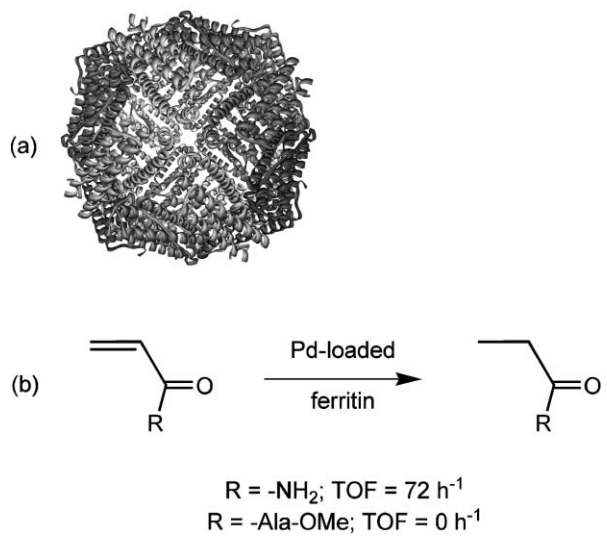

Fig. 11 (a) Structure of ferritin. (b) Examples of olefin hydrogenation catalyzed by the Pd-loaded ferritin.

Ser112Gly mutant produces preferentially the (*S*)-acetamidoalanine: 31% ee (*S*) *vs.* 31% ee (*R*) for WT SAV.

In order to test the substrate specificity, acetamidocinnamate was tested as substrate for the hydrogenation in streptavidin in the presence of **Biot–1**. This bulkier substrate

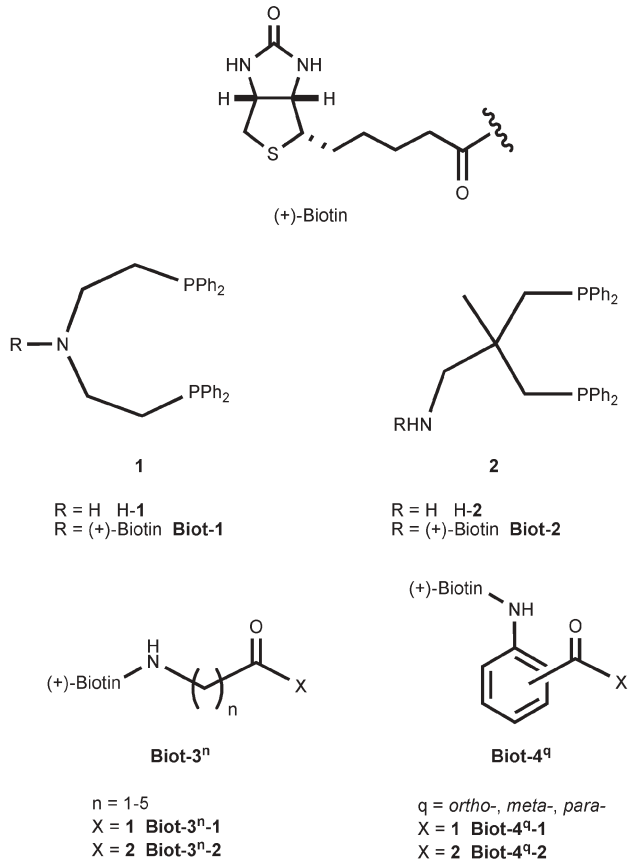

Fig. 12 Biotinylated ligands synthesized.

was hydrogenated withing 8 h at rt to produce (*R*)-acetamidophenylalanine quantitatively with an 86% ee.

Having demonstrated the beneficial effect of the second coordination sphere provided by the protein on the selectivity, we determined the influence of the host protein on the activity of the hybrid catalyst.[41] For this purpose, the tetrameric streptavidin was loaded with increasing amounts of [Rh(COD)(**Biot–1**)]$^+$ (1–10 equivalents). Beyond four equivalents of coenzyme, the optical purity of the produced acetamidoalanine steadily decreases, as the catalyst outside of the protein affords essentially racemic material. The enantioselectivity obtained was however higher that the predicted—considering that both catalytic cycles (inside- and outside the protein) proceed according to the same mechanism—a rate acceleration of three was estimated for the protein-embedded catalyst ([Rh(COD)(**Biot–1**)]$^+$ ⊂ SAV). It thus appears that the protein environment has a beneficial effect both on the *selectivity and on the activity* of these artificial metalloenzymes.[42]

3. Outlook

In the Introduction, we outlined the *pros* and *cons* of homogeneous- and enzymatic catalysis (Table 1). Having reviewed the state-of-the art in the area of enantioselective artificial metalloenzymes, we now critically discuss whether such systems indeed offer the best of both worlds.

By varying the position of the coenzyme within the chiral cavity by introduction of a spacer between the anchor and the ligand, it is possible to obtain both enantiomers of the product. In addition, genetic optimization can also lead to the inversion

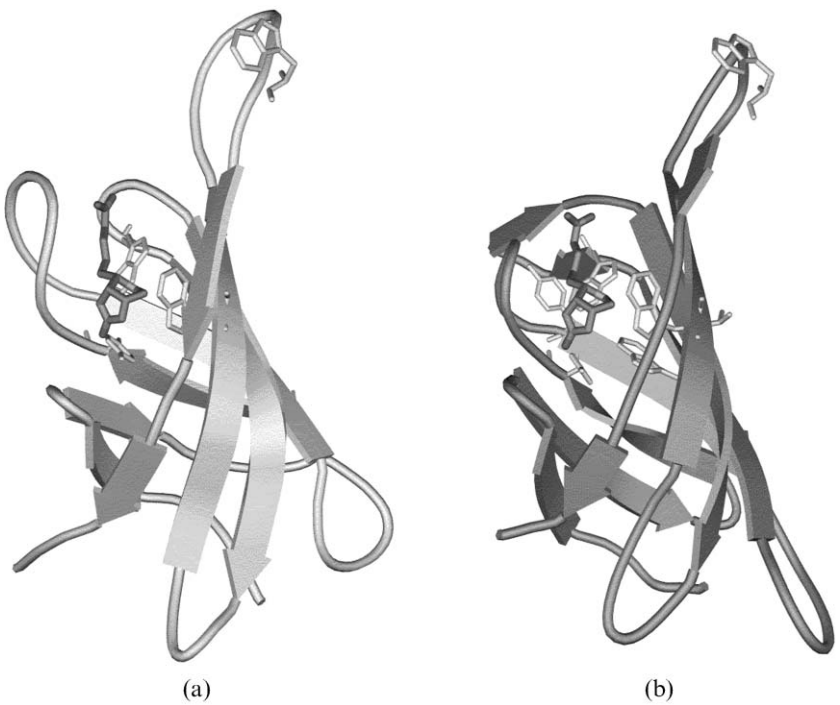

Fig. 13 Comparison of the structure of avidin (a) and streptavidin (b) (only one monomer incorporating biotin depicted; hydrophobic binding pocket residues highlighted).

of the enantioselectivity. In the hydrogenation reaction described above, using the streptavidin mutant Ser112Gly, acetamidoalanine is produced in 96% ee (*R*) using **Biot–1** in and in 57% ee (*S*) using **Biot–4^{ortho}–2** (Fig. 12).

The introduction of an organometallic catalyst precursor in a protein broadens the scope of applications of enzymes. Although enantioselective hydrogenases exist,[43] these are more typical reactions of the organometallic kingdom. To the best of our knowledge, no hydroformylation enzyme exists. Ideally, one could envisage the incorporation of any organometallic catyst (which is compatible with water) within a protein to produce a hybrid catalyst.

The introduction of a metal in a protein imposes stringent limitations on the stability of the hybrid catalyst. For example, as the protein cavity is lypophilic, we have found that oxygen traces are difficult to remove and may cause catalyst deactivation if this latter is air-sensitive. We are currently investigating different metal-catalyzed reactions where oxygen is not a problem, but the (homogeneous) catalyst dimerization is a major deactivation pathway. We expect the corresponding hybrid catalyst with isolated metal sites to display high turnover numbers as the deactivation pathway is inhibited.

The solvent compatibility of the artificial metalloenzyme is by-and-large dictated by the protein stability in organic solvents. Thus far, the research on such systems has been performed in aqueous or biphasic solutions, perhaps containing a few percent organic solvent to solubilize the organometallic species and/or the substrate.

Concerning the second coordination sphere, the production of enantiomerically enriched products, suggests that the metal coenzyme is indeed embedded in the chiral environment provided by the protein. The extent of incorporation depends on the catalyst–protein couples, and certainly deserves thorough investigation by NMR or by X-ray analysis.

As the host protein's binding pocket was by no means optimized by Nature to accomodate an enantiodiscriminating catalytic event, further developments should focus on tailoring the ''active'' site to favour a desired catalytic cycle. This may best be achieved by site-directed introduction of a suitably positionned amino acid residue with a side-chain capable of interacting either with the metal or with the prochiral substrate. These improvements may allow to render the hybrid catalysts more ''enzyme''-like in terms of substrate specificity and functional group tolerance.

As discussed above, the optimization of artificial metalloenzymes can be performed with both chemical and genetic means. Combining these powerful tools opens fascinating perspectives in the discovery and the optimization of new artificial metalloenzymes for enantioselective catalysis.

Following submission of this review, Lu and coworkers reported a Mn(Schiff-base) covalently modified apo-myoglobin for the enantioselective oxidation of thioanisole (Fig. 14).[44] Introduction of two cysteine residues (Tyr103Cys and Leu72Cys) allows to tightly anchor *via* two thioether tethers the metallo-coenzyme (which additionally interacts with His93). This dual anchoring strategy proved superior to both the mono-anchored and to the ''supramolecular'' anchoring reported by Watanabe.[34] This exciting contribution demonstrates that both covalent- and supramolecular anchoring strategies are equally suited to develop artificial metalloenzymes for enantioselective catalysis.

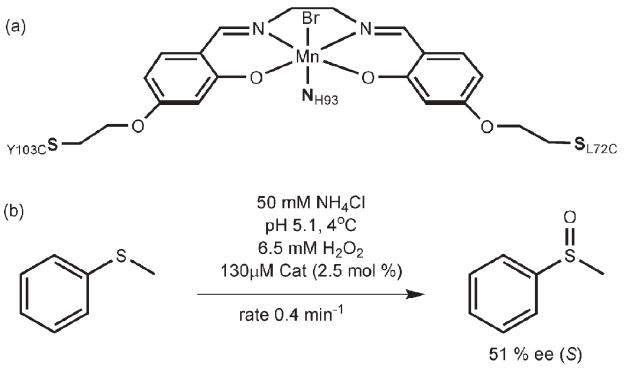

Fig. 14 (a) Dual anchoring of Mn(Schiff-base) in apo-myoglobin affords an artificial metalloenzyme for the oxidation of thioanisole. (b) Reaction conditions and yields.

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