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Review

Artificial metalloenzymes as selective catalysts in aqueous media

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Contents

Abstract

The fusion of homogeneous and enzymatic catalysis has recently drawn attention due to reported novel activities and high selectivities. The incorporation of metal-catalysts into proteins combines the advantages of both catalytic strategies. Herein we summarize recent approaches of artificial metalloenzymes applied to catalysis. The discussion includes different strategies of anchoring and screening for improved selectivity.

Keywords: Asymmetric catalysis; Artificial metalloenzymes; Hybrid catalysts; Designed evolution; Redox reactions; Hydrolysis; C–C bond formation; DNA-cleavage

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1. Introduction

Nature has provided us with a vast number of enzymes having evolved over millions of years. Starting from amino acid building blocks, complex proteins evolved to perform a variety of catalytic tasks. The scope of amino acid catalysis is limited and thus nature incorporated cofactors and/or metal-ions. It is estimated that around one third of all enzymes are metalloproteins and that some of the most difficult biological transformations are mediated by these [1]. Many enzymes show an intrinsic promiscuity [2,3] for different types of reactions whereas other activities are obtained with only small changes of the active site or the chiral environment [4]. In metalloenzymes, the diversity of promiscuous activity is increased by the variety of metallic ions that can be incorporated in the active site to catalyze a wide range of chemical transformations [5–7]. Our increased understanding of chemical and enzymatic catalysis, especially since the advent of genetic engineering and recombinant technology, has led to attempt the development of new enzymes and catalysts with modified activities, specificities and activities [4,8–10]. Only 50 years ago, the first hybrid catalyst showed promising selectivities by applying Pd on silk [11]. Optically active amines and amino acids were obtained from the corresponding alkenes by hydrogenation. Interestingly, it took 20 more years to develop the first homogeneous artificial metalloenzyme for hydrogenation [12]. Artificial metalloenzymes, as reviewed here, are hybrid catalysts resulting from the introduction of a metal-complex with catalytic activity into macromolecular hosts, such as a protein, DNA or an antibody, which provide a well-defined second coordination sphere and thus induce the selectivity of the reaction (Fig. 1). Several reviews on artificial enzymes have been recently published [1,13–25].

Herein we summarize the most recent advances in the field of artificial metalloenzymes for regio- and stereoselective catalysis with an emphasis on the reaction types implemented thus far which include redox reactions, C–C bond formation, hydrolysis and DNA-cleavage. First, the general anchoring strategies are presented, followed by a detailed description of the different reaction types. For hydrogenation reactions using the biotin–(strept)avidin technology two complementary optimization protocols are described in more detail.

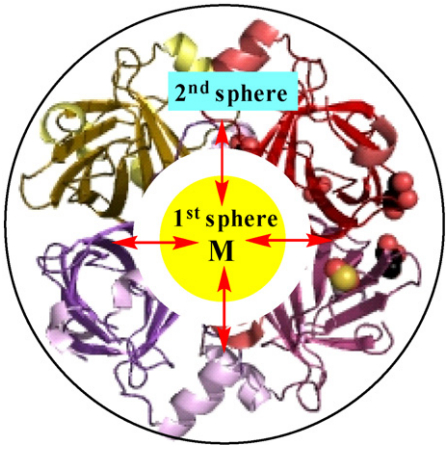

Fig. 1. Characteristics of artificial metalloenzymes: The metal–ligand complex (M) is anchored to the biomolecule host and provides the 1st coordination sphere whereas the host provides the 2nd.

2. Classification of artificial metalloenzymes

2.1. Reaction types

Artificial metalloenzymes differ from each other in three important ways which are summarized in Table 1: (a) the metal-complex, (b) the biomolecule host, and (c) the anchoring strategy.

2.2. Anchoring strategy

Many different approaches for the incorporation of the artificial catalytic moiety with the macromolecular host have been applied [1,13–24,53]: (a) supramolecular anchoring, (b) dative anchoring, and (c) covalent anchoring. Many experiments utilize the metal and the protein in a "black box" without precise knowledge of the artificial coenzyme's localization [54,87–96]. Most of these are assumed to have a supramolecular affinity for the protein whereas others are assumed to have a dative anchoring of, e.g. cysteine-residues complexed to the metal. Several examples of in vivo evolution of designed proteins for artificial metal catalysts via immunization and formation of antibodies have been reported [97]. For catalysis, the metal-complex is incorporated via supramolecular interactions with the isolated immunoglobin.

2.2.1. Supramolecular anchoring

In the context of supramolecular anchoring [22] of metal cofactors within a host protein, the transport proteins serum albumin – especially from bovine (BSA) – play a key role. These display a remarkable ability to bind a variety of hydrophobic guests tightly, including fatty acid, steroids, thyroxine, porphyrins, etc. A variety of enantioselective transformations were performed with these proteins, including sulfoxidation [89–91,98], epoxidation [93–95], reduction [87,88], and Diels–Alder cycloaddition reactions [96].

In the 1970s, Whitesides [12] pioneered the concept of introducing a homogeneous catalyst within a protein environment to guide enantioselectivity (Figs. 2, 3a). Whereas Knowles' innovative approach for organometallic catalysis consisted of providing

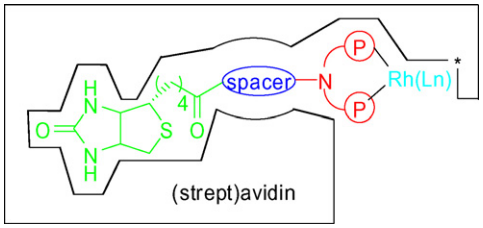

Fig. 2. Biotin–(strept)avidin technology: artificial metalloenzymes [Rh(L_n)(biotin-ligand)]⊂streptavidin for enantioselective catalysis based on the anchoring of a catalytically active metal fragment within a host protein via a ligand, a spacer and biotin.

Table 1
Reactions catalyzed by artificial metalloenzymes

Reaction type	Metal-complex	Biomolecule host	Anchoring	References
Hydrogenation	Rh-biotin	(Strept)avidin	Supramolecular	[12,26–36]
	Rh	Papain	Covalent	[34,35,37,38][a]
	Rh-diphosphine	Antibody	Supramolecular	[39]
	Pd	Apo-ferritin	Supramolecular	[40]
Ketone reduction	M-biotin: M = Ru, Ir, Rh	(Strept)avidin	Supramolecular	[41,42]
Alcohol oxidation	Zn–Cu exchange	Carboxypeptidase	Dative	[5][a]
	M-biotin: M = Ru, Ir, Rh	(Strept)avidin	Supramolecular	[43][a]
Sulfoxidation	Mn, Fe-corrole	Bovine serum albumin	Dative	[44]
	Cr, Mn–Schiff base	Myoglobin	Dative	[45–47]
	Mn–Schiff base	Myoglobin	Covalent	[48]
	MO_4^{n-}: M = V, Mo, Re, Se, W	Hydrolases, ferritin	Dative	[49–52]
Epoxidation	Mn–Schiff base	Papain	Covalent	[34,53]
	Zn–Mn exchange	Carbonic anhydrase	Dative	[6,7]
Dihydroxylation	OsO_4	Bovine serum albumin	Dative	[54]
Peroxidation	Se	Subtilisin	Covalent	[55,56][a]
	Fe-porphycene	Myoglobin	Dative	[57][a]
	Fe-hemin	DNA, RNA	Supramolecular	[58,59][a]
Hydrolysis	Cu-phenantroline	Adipocyte lipid-binding protein	Covalent	[13,60]
Diels–Alder cycloaddition	Cu-phthalocyanine	Bovine serum albumin	Dative	[36,61]
	Cu-bidendate diimines	DNA	Supramolecular	[62–64]
Hydroformylation	Rh	Human serum albumin	Dative	[65–67]
Acyl-transferase	Se	Subtilisin	Covalent	[68][a]
Transamination	Cu-pyridoxamine	Ribonuclease S	Supramolecular	[69]
DNA-cleavage	Cu-phenantroline	DNA-binding proteins	Covalent	[70,71]
	Zn	Peptide-fragment with Rh-intercalator	Covalent	[72,73]
	Eu, Ce, Cu	Helix-Turn-Helix/Ca-binding motif	Covalent	[74–78]
	Ce	Zinc finger protein	Covalent	[79,80]
	Zr	Peptide nucleic acid	Covalent	[81]
	Ce	Complementary peptide nucleic acid	Supramolecular	[82–86]

[a] No selectivity reported.

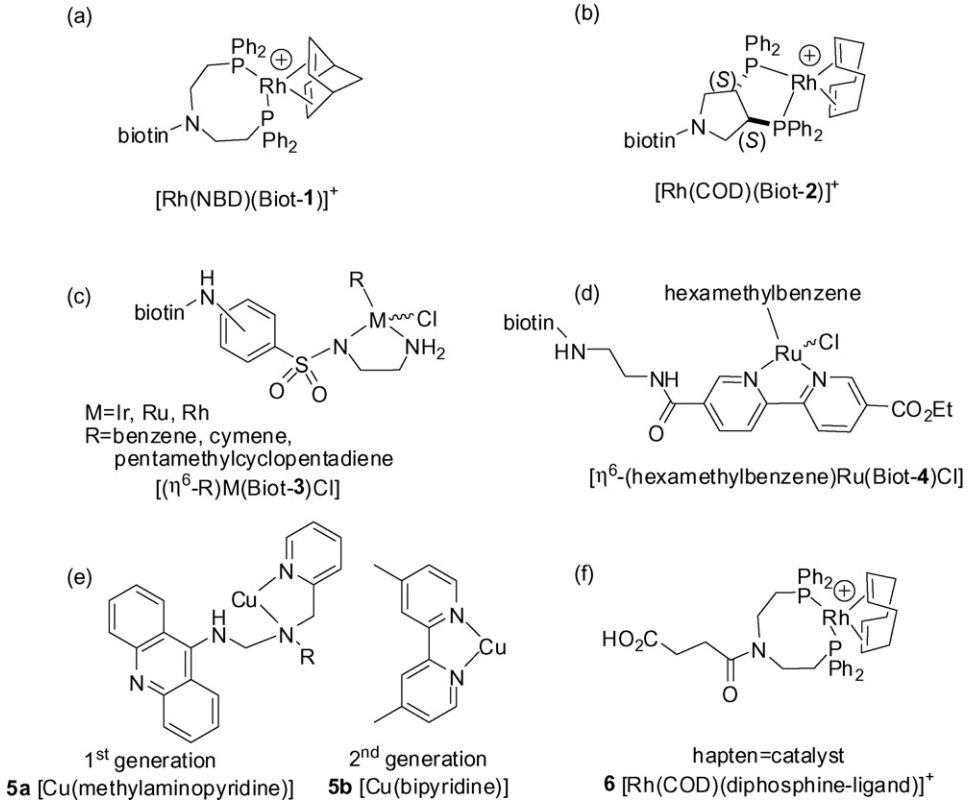

Fig. 3. Supramolecular anchoring using (a)–(d) biotin derivatives [12,26–36,41–43], (e) DNA-intercalators [64] and (f) a rhodium–diphosphine complex [39].

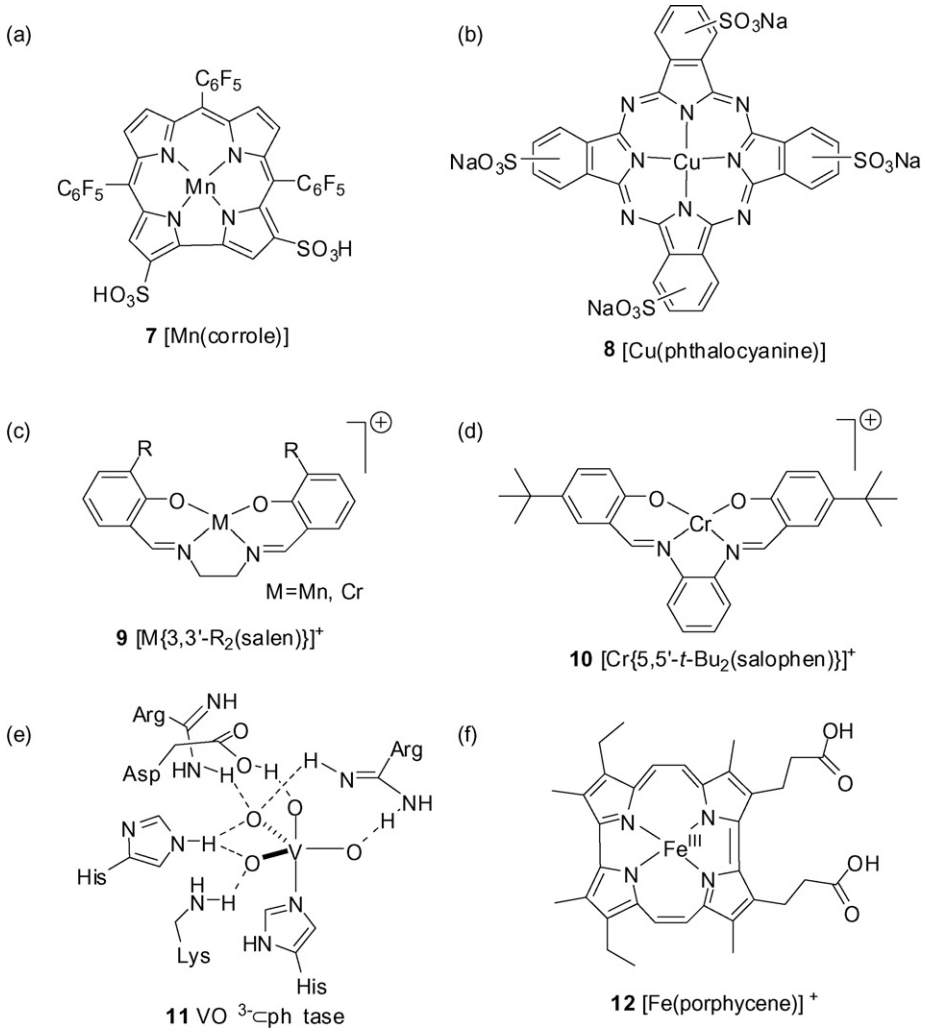

Fig. 4. Complexes for dative anchoring to biomolecules: (a) [Mn(corrole)] [44], (b) [Cu(phthalocyanine)] [36,61], (c) [M(salen)] [45–47], (d) [Cr(salophen)] [46,47], (e) VO_4^{3-}⊂phytase including the involved amino acid residues of phytase [49–52] and (f) [Fe(porphycene)] [57].

a chiral first coordination sphere to an achiral catalyst with a well defined activity to direct enantioselectivity [99], Whitesides' insight consisted in the biomimetic approach of embedding a homogeneous catalyst within a protein environment, to form a hybrid catalyst. The high affinity of biotin to the corresponding proteins (e.g. biotin–avidin K_a 10^{15} M^{-1}) has been intensively applied in the biotin–(strept)avidin technology.

Several molecules with high affinities to proteins have been utilized and are depicted in Fig. 3. Supramolecular anchoring of an achiral metal-complex within a DNA-double helix was achieved based on the intercalation of an aromatic moiety in DNA (Fig. 3e). An attractive method to obtain natively evolved proteins designed for distinct transition metal complexes is the preparation of antibodies against the chosen catalyst [39,100–103]. This area has been reviewed elsewhere [97] and thus only one recent example is presented. Harada et al. showed that immobilization with KLH-conjugated hapten **6** (KLH = keyhole limpet hemocyanin) affords monoclonal antibodies with high affinity towards hapten **6** [39]. In the presence of hapten **6**, these immunoglobulins catalyze asymmetric hydrogenation of prochiral alkenes (Fig. 3f).

2.2.2. *Dative anchoring*

Kaiser et al. were the first to modify the active site of an enzyme by dative modification to afford an artificial metalloenzyme with novel catalytic properties by substitution of zinc by copper in carboxypeptidase A (CPA) [5]. This approach was coined chemical mutation and the first report concerned oxidase activity of copper(II)⊂CPA.

As the hemin⊂HSA complex has shown to be anchored through an interaction between a tyrosine Y161 and the iron-ion [104], it is believed that Mn-corrole **7** and Cu-phthalocyanine **8** are anchored to bovine serum albumin (BSA) with this tyrosine residue as well. Several metal or metal–ligand complexes have been anchored in this fashion and some examples are depicted in Fig. 4.

2.2.3. *Covalent anchoring*

Kaiser et al. [105] demonstrated that novel catalytic functions can be created by covalent modifications of an amino acid residue with appropriately modified coenzyme analogues. A protease was successfully converted to a highly effective oxidoreductase by covalent modification of one distinct thiol group

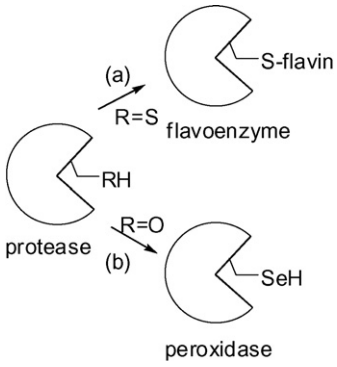

Fig. 5. Early approaches to covalent attachment of (a) flavins [105] and (b) Se [68] to obtain altered enzymatic activity.

of cysteine with flavins (Fig. 5a). A similar approach was applied by Hilvert who developed a chemical methodology for the conversion of the serine, buried within subtilisin's active site, into selenocysteine (Sec) (Fig. 5b) [55,56,68]. Thus, the hydrolytic activity of a protease was altered to novel hydrolytic [68] and redox [55,56] activities, respectively. To improve the ligation efficiency, the same group introduced a Sec-mediated native chemical ligation of peptide thioesters with Sec-containing peptide-fragments [106]. In nature, Sec was found to be post-translationally incorporated and it has been found in all life kingdoms for catalyzing mainly oxidations [107–109]. Compared to Cys, Sec displays distinct properties including: pK_a of Se-H versus S-H, redox properties, etc.

Recently, several methodologies for covalent anchoring were published by Reetz [34,36], Lu [48], Distefano [13,60], van Koten [110] and Salmain [111] (Fig. 6). Some of the disclosed metal-containing proteins do not show catalytic activity and/or selectivity. Papain [36,37], myoglobin [48] and adipocyte lipid-binding protein (ALBP) [13,60] were linked to the metal complexes via cysteine residue(s) (Fig. 6a–c, e, g) whereas for cutinase [110] and lipase [36] inhibiting properties of phosphonates were exploited to form a covalent bond between the active site and the phosphonate bearing a catalytic moiety (Fig. 6d, f). The Mn-salen complex **17** was doubly anchored to myoglobin to get a well-defined localization of the metal complex (Fig. 6e) [48]. For DNA-cleavage, a DNA binding Rh-complex was ligated to a nuclease-active Zn-complexed peptide to ensure selective binding and high activity (Fig. 6h) [72,73].

3. Reactions catalyzed by artificial metalloenzymes

A variety of different reaction types have been implemented using artificial enzymes. The borderline between artificial enzymes and synthetic catalysts is ill-defined (from which size is a peptide-fragment a biomolecule?). Several examples of metal-free artificial enzymes [54,87–96] and enzyme-mimics [112,113] have been reported and are not covered here.

3.1. Redox reactions

Redox reactions play an important role in organic chemistry and there is an increasing demand for selective and mild reaction conditions [114]. Over-oxidation or unselective conversion of multi-functionalized substrates are the main challenges next to the use of environmentally harmful solvents and toxic metals. However, there are redox reactions in classical organic chemistry which have no counterpart in nature. The combination of the enzyme-like selectivity with organometallic activity could be achieved with novel artificial metalloenzymes which are presented in the next sections.

3.1.1. Hydrogenation

From the early stage of development of artificial metalloenzymes, the hydrogenation of unfunctionalized alkenes has been intensively investigated. Wilson and Whitesides demonstrated the principle of supramolecular anchoring for the first time in the reduction of *N*-acetamidoacrylate obtaining *N*-acetamidoalanine in 41% *ee* (*S*) and quantitative conversion (Fig. 7a) [12]. In 1999, Chan et al. hydrogenated itaconic acid in the presence of an enantiopure biotinylated pyrphos-ligand (Fig. 7b) [26]. Depending on the configuration of the ligand (*S*,*S* or *R*,*R*) and the operating conditions, the *ee* of the resulting product varied between 48% (*R*) and 26% (*S*).

3.1.1.1. Designed evolution of artificial metalloenzymes. Inspired by the pioneering work of Whitesides [12], we endeavour to use the modern tools of genetic and chemical engineering to control the second coordination sphere of the homogeneous catalyst for hydrogenation [27–33]. The chemogenetic diversity matrix consists of a ligand scaffold, a spacer and genetic mutations (Fig. 8) [23]. Whereas genetic engineering has been enormously successful in elucidating and modifying enzyme mechanisms, including for the directed evolution of enantioselective enzymes, one of us was the first to show that an artificial metalloenzyme could be modified genetically [28] to improve its enantioselectivity, thus paving the path for further directed evolution of artificial metalloenzymes [35]. The general strategy that we employed initially was a "chemogenetic approach" [13], whereby both the various components of the metal complex (e.g. the metal, the 1st coordination sphere and the "spacer" linking it to the protein) as well as the protein "scaffold" were subjected to optimization. The chemogenetic approach to artificial enzymes has the potential for providing hybrid protein catalysts "made to order" [23]. Our preferred methodology is a combination of rational design and combinatorial screening leading to the "evolution" of the enzyme, for which we borrow the term *designed evolution* [115].

Designed evolution incorporates the need for rational decisions on choices of scaffolds and elements to combine, followed by several rounds of screening to perfect those elements that cannot be predicted *a priori*. It is noteworthy that the reaction conditions, such as a requirement for high temperatures or extreme of pH, may impose evolutionary restrictions on the protein scaffold and that for extensive screening it is preferable to start with an appropriately robust scaffold [116,117]. Due to the number of possible combinations of protein hosts, it is also convenient to limit the variations to a few designed choices: e.g. avidin and streptavidin. The latter provides a deeper binding pocket for the artificial metal–biotin complex. It is envis-

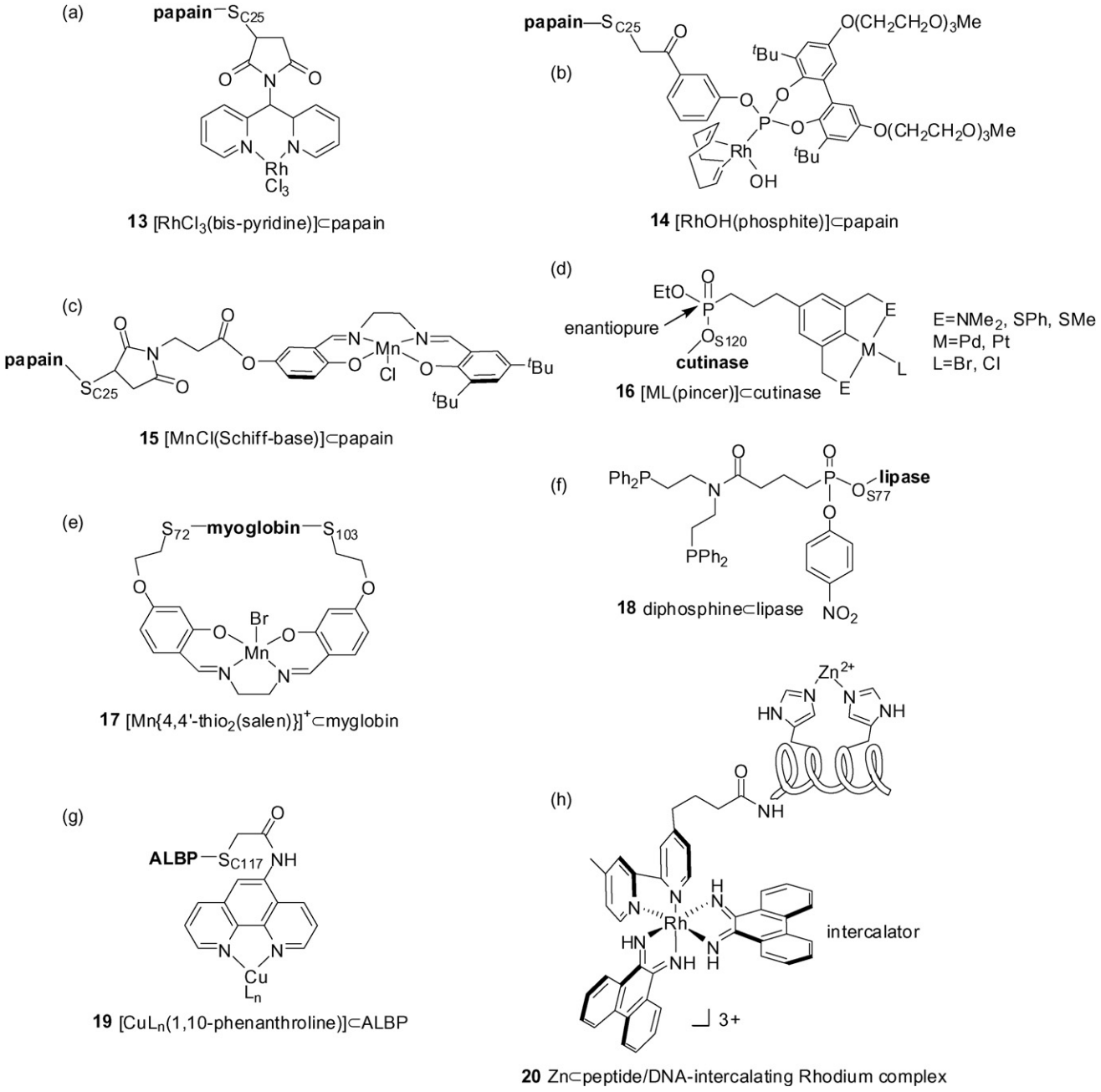

Fig. 6. Covalent anchoring of ligands and complexes to (a)–(c) papain [36,37], (d) cutinase [110], (e) double-anchoring to myoglobin [48], (f) lipase [36], (g) adipocyte lipid-binding protein (ALBP) [13,60] and (h) an DNA-intercalating peptide-rhodium complex [72,73].

aged that as we learn more about the reaction mechanisms of these particular hybrid catalysts, particularly as concerns the 2nd coordination sphere, we will be able to make wiser choices for "designed evolution", optimally leaving the really "fine-tuning" to genetic, evolutionary approaches. Using wild-type streptavidin (WT Sav) combined with biotin–ligand Biot-**1** as a starting point, two steps of evolution of the artificial enzyme (first step: chemical optimization; second step: genetic optimization) leads to reversal and improvement of enantioselectivity for the hydrogenation of α-acetamidocinnamic acid from 94% *ee* in favor of (*R*) to 88% *ee* in favor of (*S*) (Fig. 9) [29,32].

Further studies showed that introduction of chiral amino acid spacers – proline III or phenylalanine IV – between the biotin anchor and the flexible aminodiphosphine moiety **1**, combined with saturation mutagenesis at position S112 of streptavidin, affords second generation artificial hydrogenases displaying improved organic solvent tolerance, reaction rates and selectivities (≥95% *ee* for both enantiomers) (Fig. 10) [28,33].

In order to test the stability and robustness of the artificial metalloenzymes, we tested them in the presence of increasing amounts of dimethyl sulfoxide (DMSO) and ethyl acetate, the latter under biphasic conditions [31,33]. In the presence of the

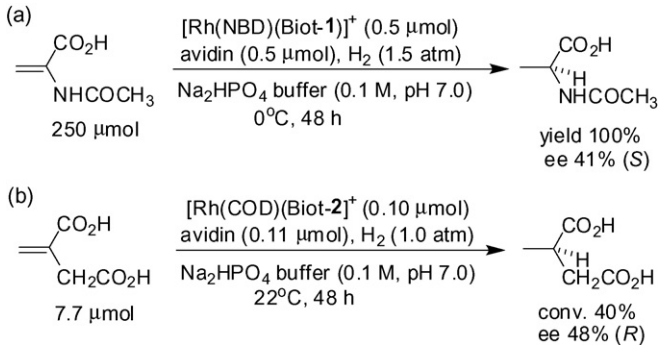

Fig. 7. Artificial metalloenzymes based on the biotin–avidin technology for the hydrogenation of alkenes; operating conditions used by (a) Whitesides [12] and (b) Chan [26].

robust spacer III, the conversion and *ee* remained high (45% DMSO: conversion 94%, *ee* 86% (*S*); 45% ethyl acetate: conversion 85%, *ee* 87 (*S*)). In strong contrast [Rh(COD)(Biot-**1**)]$^+$ (without spacer) performs poorly with the phenyl-substituted substrate (conversion <10%), whereas the terminal alkene (R = H) was converted in modest yield (<70%) under the same conditions. Recent results for the immobilization of the artificial metalloenzyme using commercially available biotin–sepharose gave comparable results to the homogeneous counterparts [Rh(COD)(Biot-**1**)]$^+$ [33].

3.1.1.2. Directed evolution of artificial metalloenzymes. The concept of directed evolution of enantioselective hybrid catalysts [34,53,118] has been applied by Reetz et al. for hydrogenation of alkene methyl esters [35,36]. Repeating cycles of random mutagenesis/expression/screening improved the catalytic profile of [Rh(COD)(Biot-**1**)]$^+$⊂WT Sav to 65% (*R*) and switched selectivity to 7% (*S*) (Fig. 11).

3.1.1.3. Papain-bound Rh-catalysts. Reetz (Fig. 12a) [34], de Vries and Feringa (Fig. 12b) [37,38] successfully attached Rh-catalysts to papain by using a maleimide linker. The catalysts were active, but displayed very little, if any, selectivity. This was argued to be due to the high flexibility of the protein's cavity. This host protein was chosen to easily accommodate ligand, metal and substrate. The choice of host is an essential strategy to ensure the accommodation of the guest-molecule but also to provide enough secondary interactions between the protein and the metal-complex to induce selectivity.

3.1.1.4. Rh-antibody catalyst. Harada et al. recently published the first example of asymmetric hydrogenation of an amino acid precursor catalyzed by the achiral complex of a transition metal combined with an immunoglobulin [39]. The resulting Rh-complex⊂antibody reduces *N*-acetamido acrylic acid to *N*-acetyl-(*S*)-alanine with an *ee* of >98% (Fig. 13). Rh⊂antibody 1G8 was found to be a stereoselective catalyst with high substrate specificity through second-sphere interactions. Interestingly, the hapten used to generate the antibodies does not incorporate a substrate mimic. However, the COD-ligand is assumed to mimic the substrate which was shown by the acceptance of the terminal alkene (R = H). The phenyl-substituted alkene was not converted, presumably as the elicited active site was too small to accommodate the larger *N*-acetamido cinnamate.

3.1.1.5. Apo-ferritin for Pd-nanoparticle formation and catalysis. Ferritin is an iron-storage protein [119]. Watanabe et al. demonstrated that this protein can form a Pd⊂apo-ferritin complex via a formation of a Pd nanoparticle in the protein capsid (inner-diameter 12 nm). Size selective olefin hydrogenation was observed at improved rates compared to the protein-free reaction (Fig. 14) [40]. However, no enantioselectivity was observed. So far, the construction of the nanoparticle within restricted interi-

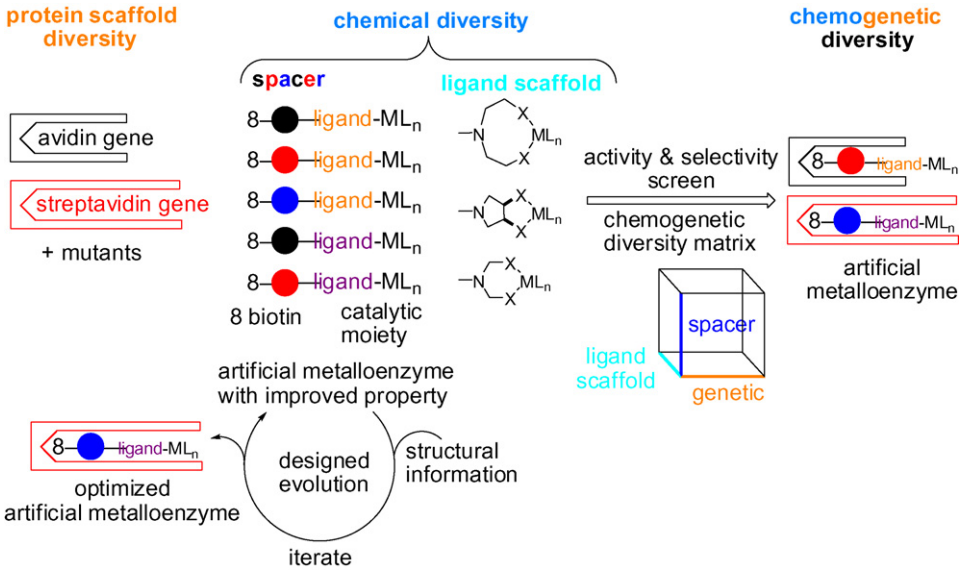

Fig. 8. Starting with a subset of appropriate protein scaffolds and chemically diverse homogeneous catalysts, a chemogenetic diversity matrix can be used to screen for improved characteristics. Designed evolution consists of iterative rounds of screening and selection of the chemogenetic diversity that is introduced, at least partially, according to the structural information available.

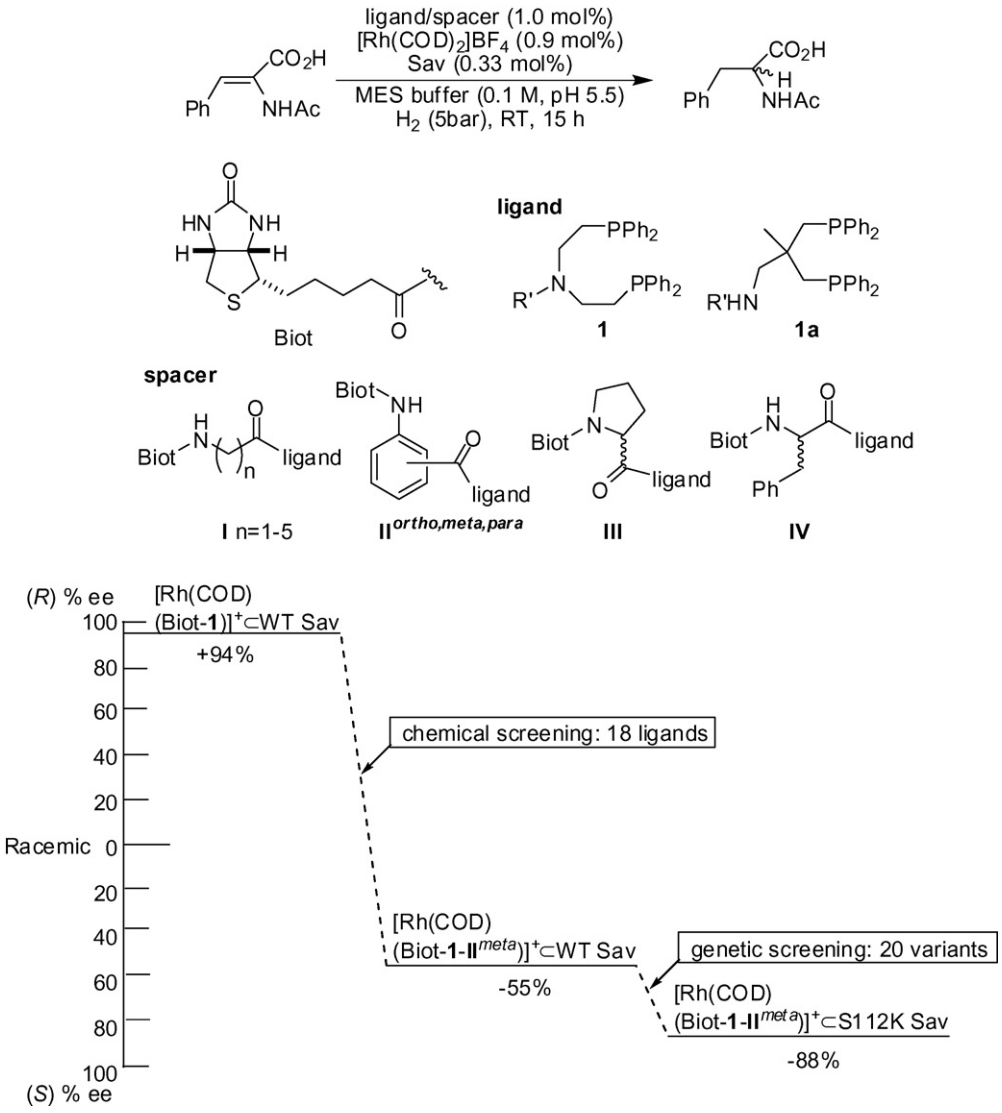

Fig. 9. Two steps of evolution of [Rh(COD)(Biot-**1**)]$^+$⊂WT Sav (first step: chemical optimization; second step: genetic optimization) [29,32].

ors of a protein assembly was the main goal. To the best of our knowledge, no attempt was made to optimize the selectivity of such nanoparticles.

3.1.2. Ketone reduction

Inspired by the findings of Sugimoto and Baba who reported on the enantioselective reduction of aromatic ketones by sodium borohydride in the presence of BSA [87,88], we focused on enantioselective transfer hydrogenation reactions catalyzed by d^6-piano stool complexes using (strept)avidin as host [41,42]. The enantioselective transfer hydrogenation of several prochiral ketones was achieved by applying [η^6-(arene)Ru(biotin-ligand)Cl]⊂streptavidin (Fig. 15) which were evolved by chemogenetic optimization (see also Fig. 7). This

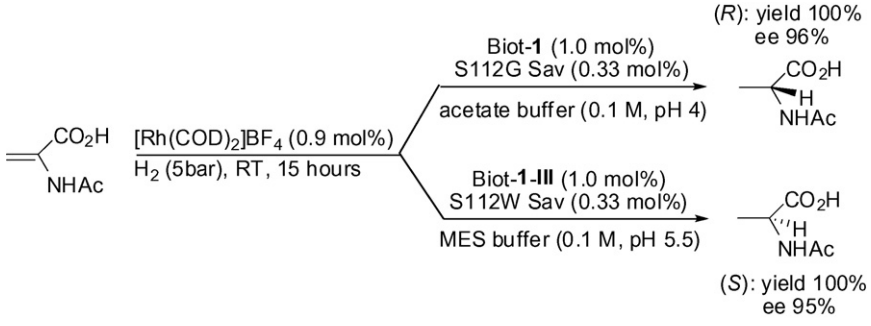

Fig. 10. Summary of selected results of the asymmetric hydrogenation improved by designed evolution [28,33].

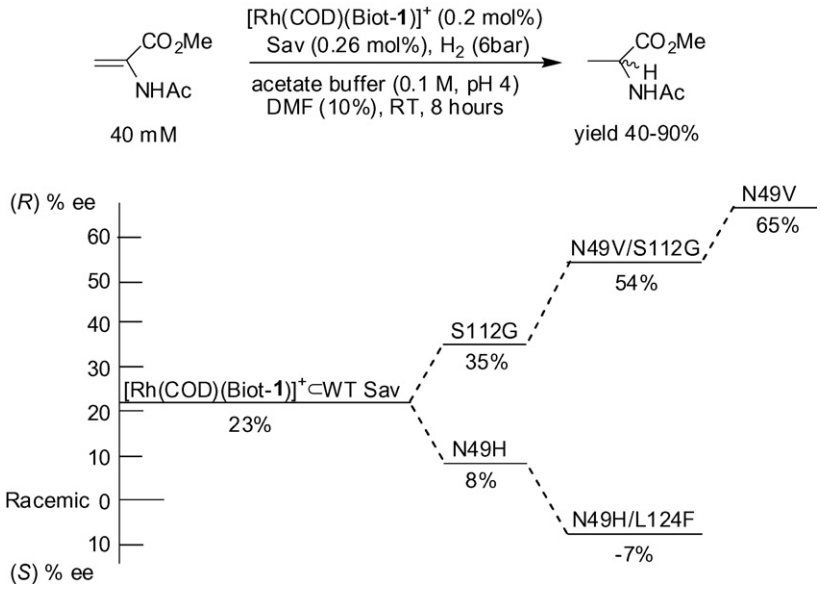

Fig. 11. Directed evolution of [Rh(COD)(Biot-**1**)]$^+$⊂WT Sav for achiral hydrogenation of alkenes [35,36].

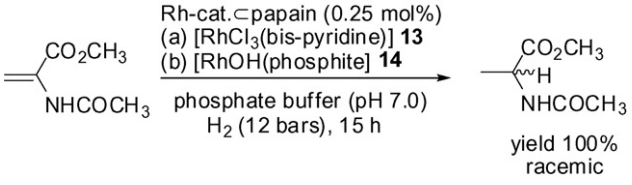

Fig. 12. Covalently anchored Rh-complexes by (a) Reetz [34] and (b) de Vries, Feringa [37,38] with papain and application in the hydrogenation of alkenes.

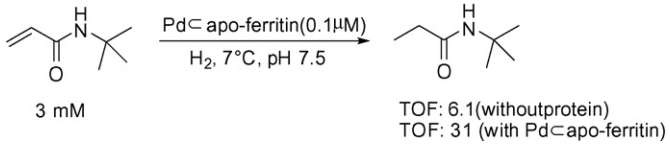

Fig. 14. Pd⊂apo-ferritin catalyzed hydrogenation of terminal alkenes [40].

highlights the strong potential of the designed evolution strategy. Remarkably, the choice of capping arene (benzene or *p*-cymene) has a strong influence on determining which enantiomer is preferentially produced.

3.1.3. Alcohol oxidation

By substituting zinc by copper in carboxypeptidase (CPA), Kaiser et al. obtained a novel oxidase activity whereas the native hydrolytic activity was diminished [5]. Ascorbic acid was oxidized to dehydroascorbic acid (Fig. 16a).

Based on a similar approach as for the transfer hydrogenation, we applied Ru, Ir and Rh-complexes (M-Biot-**3** or M-Biot-**4**) for the oxidation of racemic secondary alcohols using *tert*-butylhydroperoxide (TBHP) (Fig. 16b) [43]. Interestingly, WT avidin gave higher yield than streptavidin or mutants thereof. The protein does not suffer from oxidative damage but no kinetic resolution was observed. Having established the novel activity, our next goal is the development of selective artificial metalloenzymes for the kinetic resolution of secondary alcohols.

3.1.4. Sulfoxidation

Stereoselective sulfoxidations are challenging with classical organometallic catalysis whereas in nature a large variety of selective oxidations have been reported. Initial experiments by several groups showed that serum albumins transformed sulfides into the corresponding sulfoxides with good selectivities and good rates [89–92]. Inspired by these findings, Gross et al. contributed to the field of artificial metalloenzymes by adding amphiphilic bis-sulfonated corrole-metal complexes to various serum albumins (Fig. 17a) [44]. The resulting artificial metalloenzyme of BSA or PSA (bovine- and porcine serum albumin, respectively) gave promising selectivities for the sulfoxidation of thioanisole derivatives.

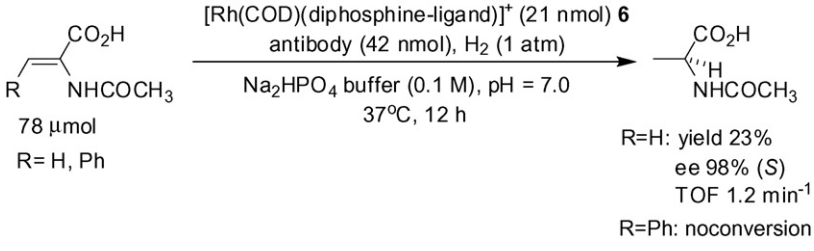

Fig. 13. [Rh(COD)(diphosphine-ligand)]$^+$⊂antibody catalyzed hydrogenation of amino acid precursors [39].

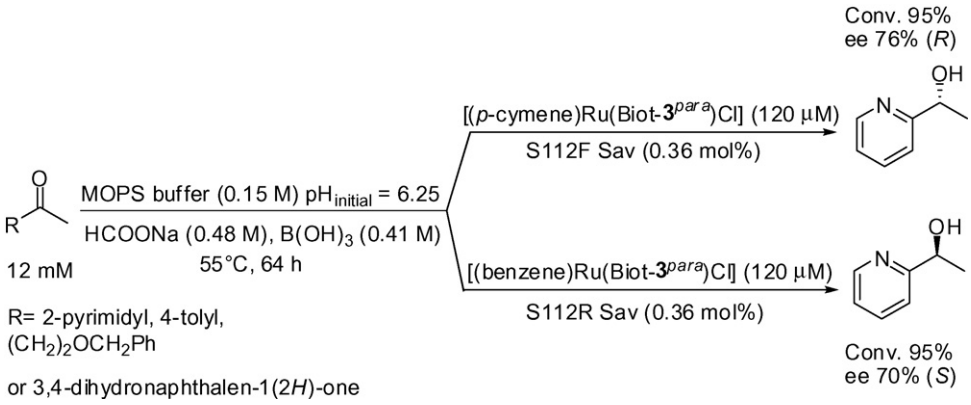

Fig. 15. Chemogenetic optimization of enantioselective artificial transfer hydrogenases [η^6-(arene)Ru(Biot-**3**)Cl]⊂streptavidin [41,42].

Myoglobin contains a heme factor which has been substituted by achiral Schiff-base–metal complexes to yield artificial metalloenzymes for enantioselective sulfoxidation. Watanabe reported on a non-covalent anchoring strategy to myoglobin mutants using chromium and manganese salen and salophen complexes with modest selectivities (Fig. 17b, (i) and (ii)) [45–47]. Interestingly, both enantiomers of the product are accessible. Lu et al. have applied a novel two-point covalent attachment strategy to obtain a more precise anchoring within the chiral protein (Fig. 17b, (iii)) [48]. The double anchoring of the artificial cofactor improved the *ee* to 51% whereas a single anchoring gave an *ee* of only 12%.

Vanadium chloroperoxidase reveals a striking similarity to the active site of phytases. Sheldon et al. investigated the sulfoxidation properties of different enzymes (acid phosphatase, apo-ferritin, phospholipase D, phytase, sulfatase) with several metalloxides (MO_4^{n-}, M: V, Mo, Re, Se, W) [49–52].

The best semisynthetic peroxidase derived from phytase and VO_4^{3-} displayed moderate enantioselectivity (up to 66%) for thioanisole derivatives in the presence of hydrogen peroxide with little detectable over-oxidation (Fig. 17c).

3.1.5. Epoxidation

Carbonic anhydrase (CA) contains zinc which catalyzes the hydration of carbon dioxide to bicarbonate. The substitution of the active-site zinc of the CA from bovine (BCA) with manganese gave an artificial metalloenzymes with a novel peroxidase activity via a bicarbonate-dependent mechanism [6,7]. Enantioselective epoxidation of styrene-derivatives was achieved with moderate selectivities but low yield (Fig. 18a). No formation of aldehyde side-products was detected. The low yield is explained by the metalloenzyme-degradation during the epoxidation. Soumillion et al. tested bovine and human carbonic anhydrase containing manganese instead of zinc [7]. The human CA (HCA) gave significantly higher yields possibly thanks to its higher stability (Fig. 18b).

Reetz reported on preliminary experiments of a covalently anchored Schiff-base–manganese metalloenzyme **15** in papain for epoxidation. Very low *ee*'s were detected [34,53].

3.1.6. Dihydroxylation

In 1983, Kokubo et al. reported the first enantioselective artificial metalloenzyme based on the dative anchoring strategy [54]. Osmium tetroxide was incorporated into BSA in a 1:1 ratio and remarkable dihydroxylation properties were obtained (Fig. 19). The anchoring is suggested to occur via two dative bonds to lysine residues within BSA's hydrophobic pocket. However, the localization of the metal is ill-defined and thus, no optimization of this process was reported.

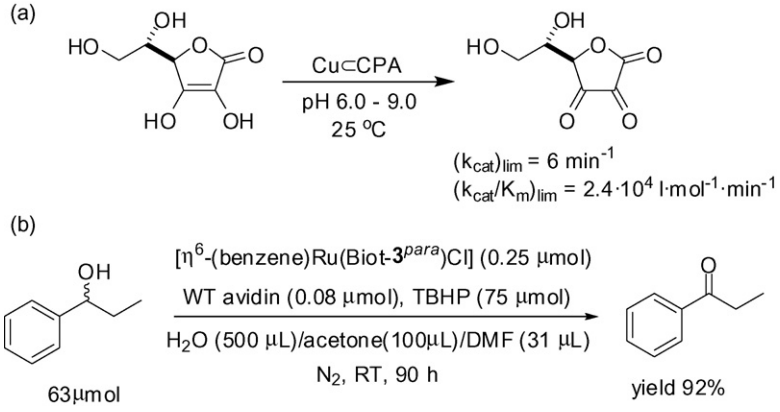

Fig. 16. Oxidation of secondary alcohols applying (a) Cu⊂CPA) [5] and (b) [η^6-(benzene)Ru(Biot-**3**^{para})Cl]⊂avidin [43].

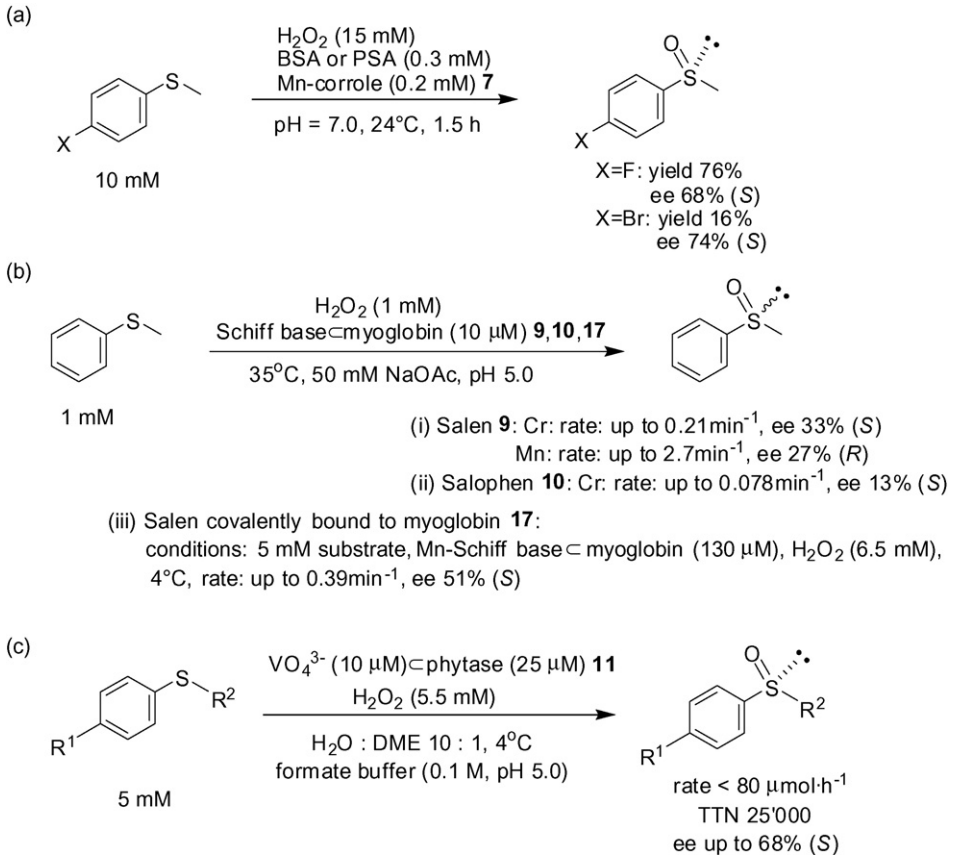

Fig. 17. Enantioselective sulfoxidation of thioanisole derivatives in the presence of hydrogen peroxide utilizing (a) [Mn(corrole)]⊂albumin [44], (b) [Mn(Schiff-base(i–iii))]⊂myoglobin mutants: (i) salen [45–47], (ii) salophen [46,47], (iii) double-anchoring salen [48], and (c) vanadium⊂phytase [49-52].

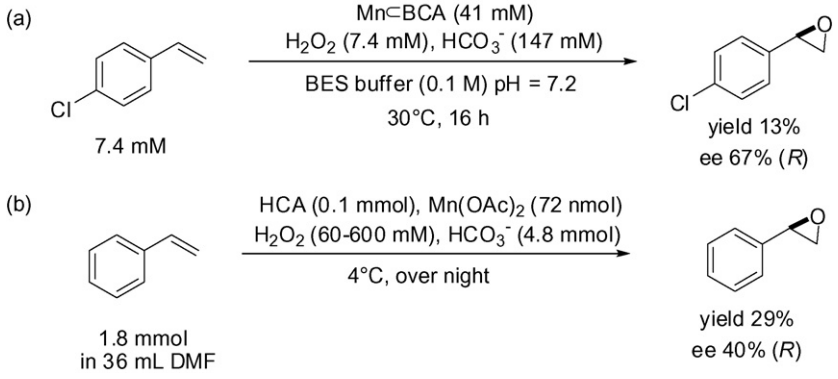

Fig. 18. Asymmetric epoxidation catalyzed by (a) Mn⊂BCA [6] and (b) Mn⊂HCA [7].

3.1.7. Peroxidation

Peroxidases are enzymes that catalyze the oxidation of inorganic and organic substrates in the presence of various peroxides (ROOH: R = H, alkyl, acyl). Several artificial peroxidases have been reported but their synthetic potential has not been exploited fully. Starting with the pioneering work on selenosubtilisin by Hilvert et al. [55,68], artificial peroxidases and their mimics have been reported recently. Hayashi [57] reported on an iron-porphycene⊂myoglobin peroxidase (Fig. 20) similar to the complex investigated for sulfoxidation by Watanabe [45–47] and Lu [48].

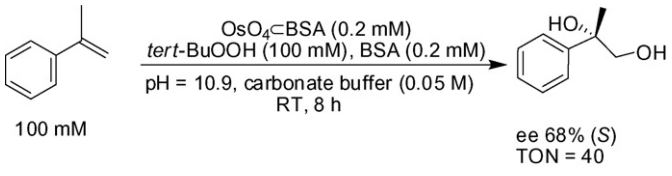

Fig. 19. Artificial enantioselective dihydroxylase based on the dative anchoring of OsO_4⊂BSA [54].

Sen et al. reported on DNA [58] and RNA [59] enhanced peroxidase activity of a hemin⊂DNA aptamer. These artificial metalloenzymes are promising tools to elucidate the structural and functional requirements of peroxidase enzymes in general.

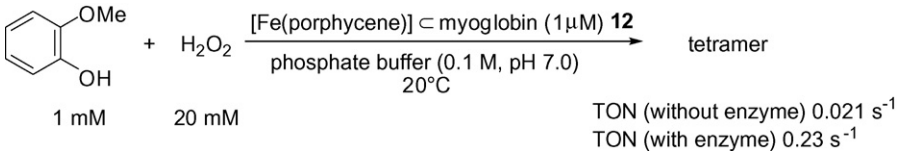

Fig. 20. Artificial peroxidase [Fe-(porphycene)]⊂myoglobin tested for oxidation [57].

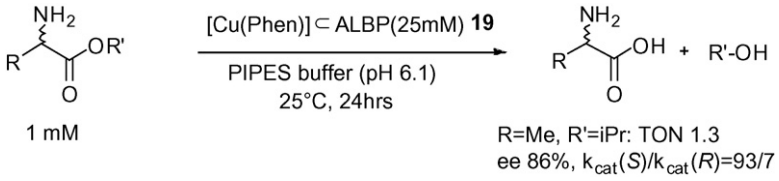

Fig. 21. Enantioselective hydrolysis of amino acid ester derivatives using [Cu(Phen)]⊂ALBP [13,60].

They might be screened in the near future for potential catalytic stereoselective oxidation reactions.

3.2. Hydrolysis

Distefano et al. reported the first enantioselective metalloenzymes with covalent anchoring of a Cu-phenantroline complex with an adipocyte lipid-binding protein (ALBP) for the hydrolysis of several inactivated amino acid esters and amides (Fig. 21) [13,60]. This study displays good kinetic resolution properties but the turnover numbers leave room for improvement. Many enzymes are known to catalyze this reaction with high activity and selectivity. However, the ALBP cavity promises to be a useful system for the development of new catalysts but strangely no one has taken up this idea for other reaction types. We hypothesize that the covalent anchoring strategy of metal-containing catalysts raises several challenges which remain to be solved.

3.3. C–C bond forming artificial metalloenzymes

Only a few examples of stereoselective C–C bond forming reactions with artificial metalloenzymes have been published to date.

3.3.1. Diels–Alder cycloaddition

Using BSA, Colonna et al. showed for the first time a protein-catalyzed stereoselective Diels–Alder reaction [96]. This concept was improved by Reetz et al. who recently reported on Cu-phthalocyanine catalyzed Diels–Alder reactions of azachalcones with cyclopentadiene in aqueous media in the presence of serum albumins, leading to the Diels–Alder adducts with high enantiopurities of up to 98% of the *endo*-product (Fig. 22a) [36,61].

A novel DNA-based asymmetric catalysis concept was introduced based on the modular assembly of a DNA-based hybrid catalyst with a supramolecular attachment of the chiral biomolecule and the achiral metal-complex by Feringa and Roelfes [62–64]. This catalytic ensemble comprises an intercalator and a copper-chelating moiety. In the first generation, these two moieties were linked via a spacer (**5a**) whereas in the second generation both sites are integrated into a single bipyridine moiety (**5b**) and thus the reactive copper centre is brought into closer contact with the chiral environment. The second generation gave an increased enantioselectivity in the catalyzed Diels–Alder reaction (Fig. 22b).

Artificial Diels-Alderases are promising targets with a high potential for synthetic applications as there are only

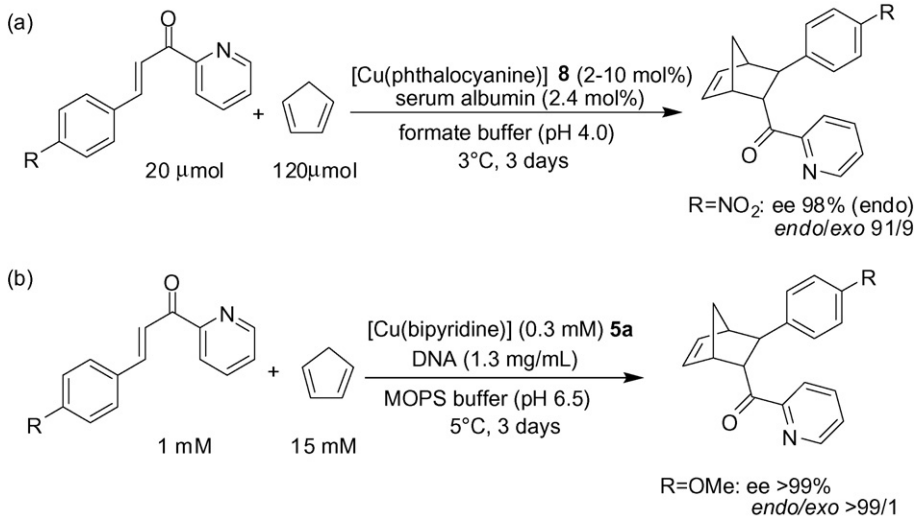

Fig. 22. Asymmetric Diels–Alder reaction catalyzed by (a) [Cu(phthalocyanine)]⊂serum albumin [36,61] and (b) Cu-ligand⊂DNA [62–64].

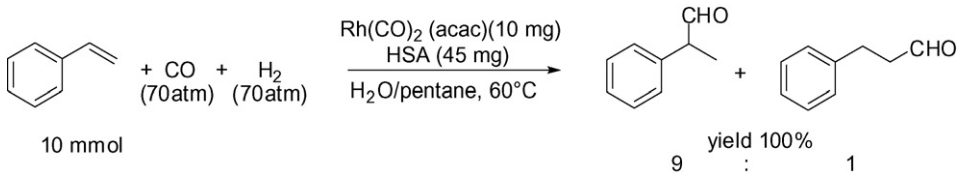

Fig. 23. Regioselective hydroformylation of styrene using a Rh⊂HSA hybrid catalyst [65,66].

few enzymatic counterparts known so far [120]. During the review process of this article another approach to a minimal artificial Diels-Alderase based on Cu-monopetide was released [121]. However, the monopeptide can hardly be called biomolecule.

3.3.2. Hydroformylation

Marchetti et al. loaded human serum albumin (HSA) with rhodium and reported the hydroformylation of styrene under biphasic conditions [65,66]. Although no anchoring of the Rh-complex to the protein was postulated, they reported high turnover numbers (>500,000) and good regioselectivities (9:1 in favor of the branched product, no enantioselectivity mentioned) (Fig. 23). Recently, the interactions between HSA and Rh-complex were suggested to be of dative origin between the metal and cysteine-residues [67]. The attractiveness of this process is the easy workup starting from gases and a volatile substrate.

3.4. Transamination

Roy and Imperali reported on a novel transaminase activity of semisynthetic ribonuclease S (RNaseS) proteins incorporating a pyridoxamine phosphate moiety [69]. This was achieved by supramolecular incorporation of the heterocyclic functionality of pyridoxamine into the C-peptide derived from RNaseS. The catalytic copper-assisted transamination of pyruvate to alanine was achieved by adding phenylalanine for the recycling of the formed pyridoxal to pyridoxamine (Fig. 24). Upon addition of phenylalanine, the reaction rate was decreased compared to the non-catalyzed reaction but chiral induction up to 31% (*R*) was observed.

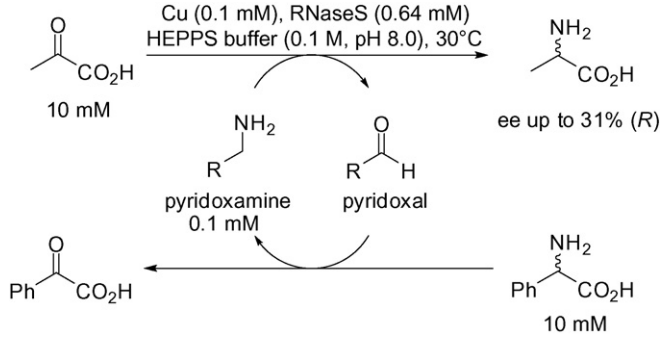

Fig. 24. Enantioselective transamination of pyruvate and recycling of the catalyst by oxidizing phenylalanine [69].

3.5. DNA-cleavage

Artificial metallonucleases are promising candidates for application in cancer therapy and have been reviewed recently [71,122–125]. DNA-sequence selective down-regulation of genes in tumerous tissues and expanding the set of recognizable DNA sequences beyond the short sequences of currently available nucleases have been the main targets of interest. Indeed, most restriction enzymes can recognize DNA sequences of up to eight base pairs. For gene therapy, the recognition of longer DNA fragments is necessary to suppress unwanted DNA scission. In 1992, Schepartz et al. reported on a covalent anchoring of [Fe(EDTA)] complex to a staphylococcal nuclease [126]. Upon addition of the iron reductant ascorbate, [Fe(EDTA)]⊂nuclease underwent a protein self-cleavage reaction mediated by reactive oxygen species.

Recently, Sigman published a catalytic oxidative DNA scission of a copper-1,10-phenanthroline complex [70,71]. However, to obtain selective and efficient DNA-cleavage both DNA-recognition and the reactive element must be integrated into one (bio)molecule. In nature, the former is accomplished by transcription factors and repressor proteins, the latter by the hydrolytic activity metal-ions such as Ca, Mg and Zn. Recent studies of artificial nucleases suggest that lanthanides (Eu, Ce) [74–77,79,80,82–86] and transition metals (copper [78], zirconium [81]) are promising metals combined with peptide motifs such as helix-turn-helix (HTH) chimera [74–76,78], zinc fingers [79,80] or peptide nucleic acids [81].

Barton et al. appended a peptide-fragment onto a ligand of a rhodium metallointercalator which binds in the major groove of DNA with high affinity [72,73]. The tethered peptide **20** binds divalent cations through two histidine residues to promote reaction at the DNA backbone.

Franklin et al. synthesized a chimeric metallopeptide with two structurally superimposable motifs (DNA recognition HTH and calcium-binding motifs) [74–78]. The oligopeptide binds to a specific DNA-sequence and to the metals Eu(III) or Ce(IV) and thereby projects the hydrolytic metal towards the backbone of DNA for cleavage. The regioselective cleavage gives only 3′-OPO_3 termini. Additionally, these artificial nucleases cleave single-stranded DNA faster than free metal-ions.

An elegant catalytic example of an artificial DNA-nuclease involving a biomolecule for recognition and a metal for activity was published by Komiyama [82–86]. This concept was termed antisense approach due to the complementarity of the biomolecule to the substrate. Komiyama et al. achieved selective DNA-cleavage by applying two modified pseudo-complementary peptide nucleic acids (pcPNA) together with

Table 2
Typical features of enzymatic, homogeneous and artificial metalloenzyme catalysis

	Enzymes	Homogeneous catalysts	Artificial metalloenzymes
Enantiomers	Single	Both	Both
Reaction conditions	Mild	Harsh	Mild
Substrate scope	Limited	Large	Large
Functional group tolerance (regioselectivity)	Large	Small	Small
Typical substrates	Flexible	Apolar	Flexible
Reaction repertoire	Small	Large	Large
Solvent compatibility	Aqueous > organic	Organic > aqueous	Aqueous > organic
Optimization	Genetic	Chemical	Chemogenetic
Second coordination sphere	Well-defined	Ill-defined	Well-defined
Recycling	Immobilization protocols	Rare	Promising
Turnover numbers	Large	Modest	Modest

Ce/EDTA cutters and he termed them artificial restriction DNA cutters (ARCUTs). The complementarity of the pcPNA to the target DNA-sequence is disturbed by the replacement of some artificial bases which enhance the invasion into the double-stranded DNA. The double-strand DNA is uncoiled forming single stranded DNA which is cleaved by a completely hydrolytic character of the Ce/EDTA-cutters with high regioselectivity.

4. Conclusion

In recent years, the field of artificial metalloenzymes has evolved from a "black box" catalyst to controlled-anchoring and more demanding systems such as selective DNA-cleavage or a variety of oxidation reactions which cannot be achieved with classical organic protocols. Homogeneous and enzymatic catalysis are widely studied with complementary strategies of development (Table 2). Enzymes are improved by a limited number of protocols with the disadvantages of that only similar reaction types are accessible, the time of development is long and only in a few cases all stereoisomers of the desired product are accessible. Directed evolution offers a powerful means to overcome the major limitations of enzymes, except perhaps the creation of novel activities. Why not copy nature in an earlier stage of catalyst development? Nature chose to incorporate cofactors and metal-ions to broaden the reaction-scope of the limited amino acid catalysis of its native protein-machinery called enzymes.

The limitation of homogeneous catalysis caused by an insufficient control of the second coordination sphere or the environmentally unfriendly protocols can be solved by a chiral environment provided by biomolecule host under mild conditions in aqueous solution. This highly interdisciplinary field is ready for more intense studies of new activities, selectivities, screening systems and engineering of these sensitive but highly promising protocols.

Acknowledgments

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