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Artificial Metalloenzymes for Hydrogenation and Transfer Hydrogenation Reactions

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6.1 Impact of Metallohydrogenases in the Field of Artificial Metalloenzymes

Artificial metalloenzyme-catalyzed hydrogenation and transfer hydrogenation reactions are among the most widely studied reactions to probe the capabilities of the protein environment in a metalloprotein hybrid to induce enantioselectivity in known transition metal-catalyzed reactions. Initially reported in the year 1978, hydrogenation reactions with semisynthetic, bioconjugated catalysts were not revisited until the beginning of the twenty-first century. Following a rapid advance in a span of 5 years, the use of artificial metalloenzymes in hydrogenation reactions has consolidated the ability to achieve catalytic activity through metal-protein cooperativity and to optimize both the catalytic activity and selectivity of metal-protein conjugates by rational chemocatalyst design, optimization of the conjugation methodology, and (biochemical) protein modification. The successful achievements in catalytic activity and product enantioselectivity displayed by metal-containing artificial hydrogenases (AHases) and artificial transfer hydrogenases (ATHases) during this short period of time have motivated researchers in the field of catalysis to further explore other catalytic reactions using artificial metalloenzymes, using both the hybridization techniques initially applied for rhodium and ruthenium metallocenters and the optimization strategies aimed at selectivity enhancement, such as site-directed mutagenesis or cofactor linker length variation. Notably, since the end of the twentieth century, a number of examples on artificial metalloenzymes for other types of transformations had been reported. Nevertheless, any literature review on the general topic of artificial metalloenzymes cannot omit the protagonist role that hydrogenation reactions have played in this field.

Hydrogenation reactions have become exceptionally popular to promote enantioselective catalysis exploiting metal-protein cooperativity because it allows the conversion of a non-chiral substrate, for example, olefins, ketones, or

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imines, toward a chiral product, involving the addition of another non-chiral substrate, hydrogen gas, which is also a non-sterically demanding reactant. The three-dimensional steric environment of the metal-protein structure plays a fundamental role in the resulting enantiomeric excess (*ee*) in such catalytic transformations.

Another attractive feature of the development of artificial metalloenzymes for hydrogenation reactions is the possibility of benefiting from the combination of proteins with strictly nonbiological metal centers. By the end of the twentieth century, platinum group metals were in the center of homogeneous catalysis and organometallic chemistry, with (enantioselective) hydrogenation as one of most prominent applications. Interestingly, none of the platinum group metals are known to occur in living organisms.

A third feature is the well-known and early pioneering work of Wilson and Whitesides on converting a protein into an artificial metalloenzyme [1]. Their work established that product enantioselectivity (initially modest) can be achieved, thereby providing a crucial starting point for later endeavors in the development of enantioselective artificial metalloenzymes, some 20 years later (Figure 6.1).

This chapter presents a comprehensive overview of AHases and ATHases. The examples discussed here will highlight the hybridization strategies that have allowed for the creation of single site-directed conjugates, the achievements and strategies in activity and (enantio)selectivity optimization, the nature of the

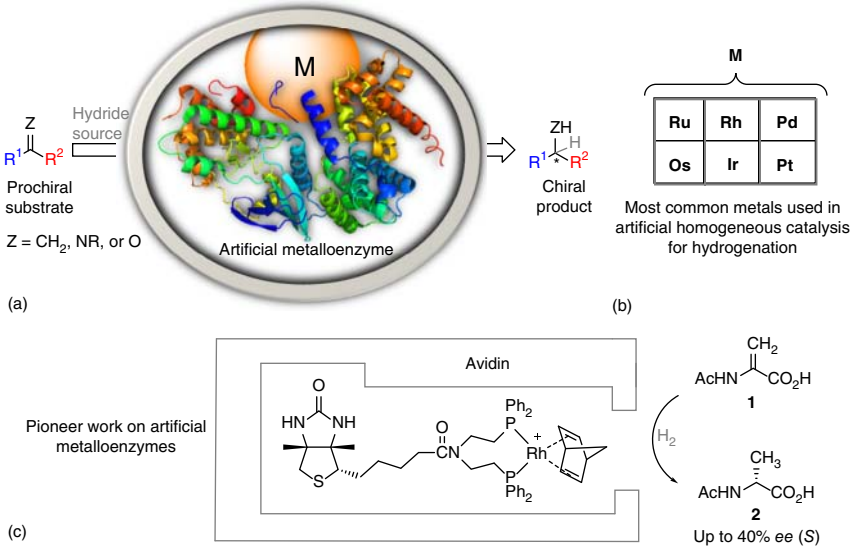

Figure 6.1 Key features that launched hydrogenation as protagonist application in the development of artificial metalloenzymes: (a) the catalyst-promoted formation of chiral products from non-chiral olefins, ketones, and imines, (b) the combination of proteins with biologically non-occurring metals, and (c) the pioneer work from 1978 by Whitesides on the conversion of a protein to a homogeneous asymmetric hydrogenation catalyst. (Wilson and Whitesides 1978 [1].)

low molecular weight chemocatalysts that have been used, and the different reactions that have been catalyzed.

6.2 Biotinylated Metal Complexes in Avidin and Streptavidin

6.2.1 Hydrogenation of *N*-Protected Amino Acids

Biotin, also known as vitamin B7, is a naturally occurring small compound composed of a valeric acid and a thiophene–urea fused bicyclic moiety. It is involved in the metabolism of carbohydrates, amino acids, and fatty acids, unless scavenged by the protein avidin present in raw egg, which is a basic tetrameric glycoprotein with twofold symmetry. The biotin–avidin interaction has a dissociation constant of 10^{-15} mol L^{-1}, making it one of the strongest supramolecular interactions known to date [2]. In biotin, the terminal carboxylic group allows for functionalization of the valeric moiety, mainly via esterification or amidation. As introduced earlier, Whitesides has studied a biotin derivative furnished with a [Rh(diphosphine)(nbd)]Tf (nbd = norbornadiene, Tf = triflate) complex (Figure 6.2) [1]. Mixtures of the Rh–biotin complex with bovine serum albumin (BSA), lysozyme, or human carbonic anhydrase (HCA) resulted in a decrease in catalytic activity of the metallocatalyst in the hydrogenation of α-acetamidoacrylic acid (**1**) in comparison with the reaction without any protein present. The deactivation of the catalyst could result from nonspecific coordination of the metallic center with the protein component. However, a 1 : 1 mixture of the Rh–biotin complex with avidin resulted in the complete hydrogenation of **1** toward acetylalanine **2**, with slightly higher turnover numbers than the reaction in the absence of a protein host (>500 vs 475, respectively) and with *ee* toward the (*S*)-product (*ee* = 41%). Since only the bicyclic moiety of biotin is involved in its strong binding with avidin, the Rh–biotin–avidin structure is able to accommodate the metallic functionality in proximity of the binding cavity, yet

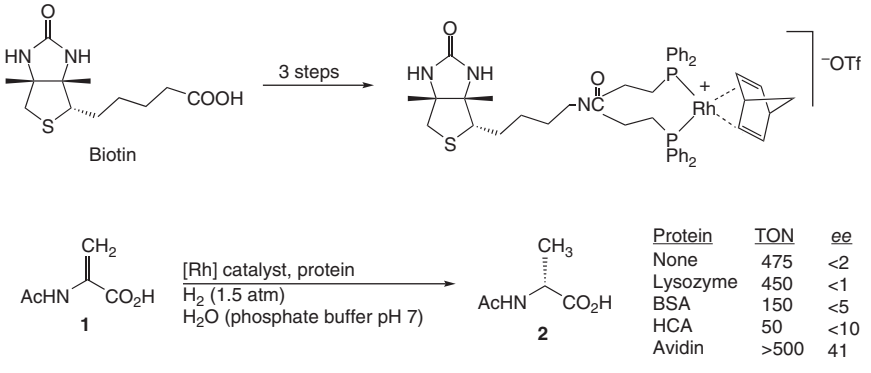

Figure 6.2 A rhodium–biotin catalyst promotes enantioselectivity in the hydrogenation of **1** in the presence of the protein avidin as observed in the enantiomeric excess (*ee*) measured by polarimetry.

keeping it accessible to the olefinic substrate. Notably, in a different experiment, when the binding cavity of avidin was initially blocked with non-metalated biotin, no *ee* was found in the reduction of **1** by the avidin–Rh–biotin mixture. Although only modest *ee*'s were obtained with the Rh–biotin–avidin hybrid, important conclusions can be derived from this work: (i) it is possible to carry out olefin hydrogenation reactions with homogeneous rhodium catalysts in aqueous mixtures in the presence of proteins, (ii) the mutual protein–metal interactions in the proximity of the avidin binding cavity do not deactivate each other, and (iii) product enantioselectivity is achieved only with the proper combination of metal fragment and protein.

Although biotin itself bears an (*S*)-stereocenter, its combination with rhodium results in the racemic hydrogenation of **1** when it is not combined with avidin. The exertion of enantioselectivity by the avidin–protein scaffold became even more evident in the work of Chan and coworkers, who functionalized biotin with the enantiopure diphosphine pyrphos and coordinated it to $[Rh(cod)_2]BF_4$ (cod = 1,5-cyclooctadiene) (Figure 6.3) [3]. The resulting enantiopure Rh(biotin-pyrphos) complex led to the hydrogenation of itaconic acid (**3**), yielding 2-methylsuccinic acid (**4**) with the same absolute configuration as the chiral ligand employed in only 11% *ee*. When the enantiopure Rh(biotin-pyrphos) catalyst was used in combination with avidin, not only was the opposite product configuration found in excess, but the observed *ee* was also higher than in the absence of avidin. Through a screening of reaction conditions, such as hydrogen pressure, pH value, and temperature, the authors were able to achieve quantitative conversions and *ee*'s of almost 50% [3]. This phenomenon of reverse enantioselectivity by a catalyst–protein combination with respect to the chirality of the catalyst fragment suggests that the selectivity is a result of protein-metallocatalyst cooperativity, and it is not promoted solely by the protein scaffold or the metallocatalyst. Indeed, using the opposite catalyst isomer resulted in the formation of the opposite product enantiomer in excess, both in the presence and absence of avidin.

This indirect evidence of close interactions of the metallic catalyst and the chemical surroundings provided by the protein scaffold, that is, the second coordination sphere, having an effect on the stability or accessibility of catalytic

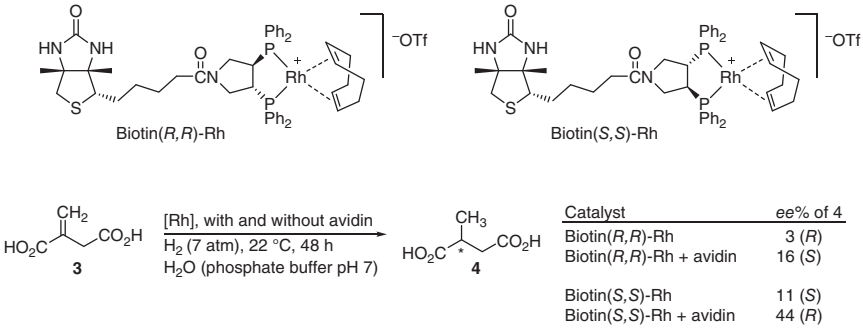

Figure 6.3 Avidin as protein host not only promotes the catalytic enantioselectivity but can also stir the configuration of the product, showing metal-protein cooperativity in the asymmetric hydrogenation of itaconic acid.

intermediates, makes avidin a protein host candidate to be subjected to *directed evolution optimization*. Originally suggested by Reetz as a strategy to enhance the enantioselectivity of enzymes and to develop artificial enzymes [4], this catalytic optimization strategy is based on the generation of mutated protein hosts as a means to change the catalytic selectivity of the resulting metalloprotein hybrid. By sequential mutations of avidin, monitoring the resulting yield and *ee* in the hydrogenation of **1** catalyzed by each metalloprotein variant, continuous iterative mutations out of positive hits lead to artificial metalloenzymes with enhanced enantioselectivities. The formal Darwinian application of Reetz's hypothesis would be addressed by his group and reported a few years later (see following text) [5]; nonetheless, the insights of Whitesides and Reetz paved the way for the extensive work by Ward and coworkers on the development of artificial metalloenzymes based on the biotin–avidin technology.

The Ward group originally studied wild type (WT) and mutants of both avidin and streptavidin (Sav) as protein scaffolds for hydrogenation catalysts. Sav is a protein isolated from the bacteria *Streptomyces avidinii*; it is unrelated to avidin and has only 30% of similarity to avidin in its primary structures. Sav does have a similar tetramer symmetry and a high affinity for biotin, though with a dissociation constant of 10^{-14} mol L^{-1} [6]. In an early study, Ward and coworkers reported on the hydrogenation of **1** using avidin, two variants of Sav and biotinylated [Rh(diphosphine)(cod)]BF_4 complexes with different organic linkers on the valeric tether (see Figure 6.4) [7]. The outcome of their study showed that (i) Sav promotes product enantioselectivity to a larger extent than avidin; (ii) larger flexibility of the linker between the biotin moiety and the diphosphine ligand allows for improved catalytic activity, albeit a detriment on enantioselectivity; (iii) avidin-based metalloenzymes perform best in neutral

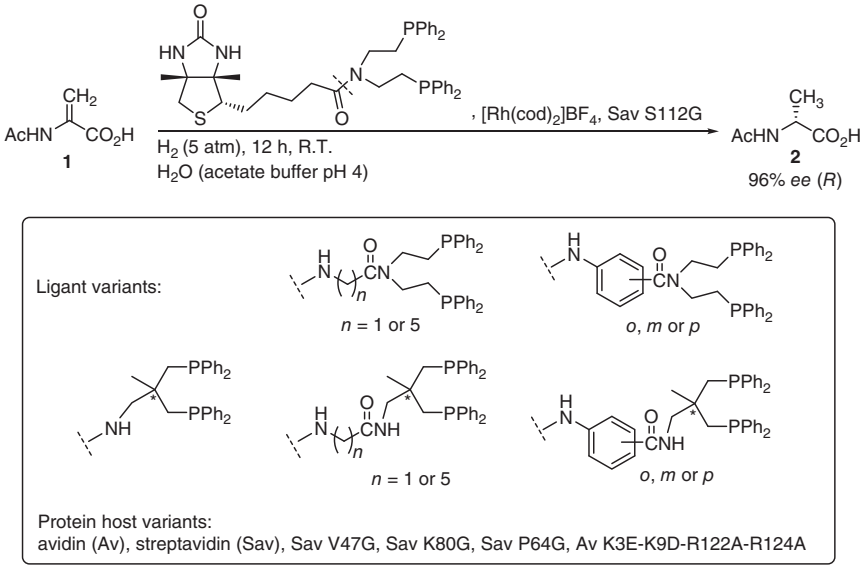

Figure 6.4 Series of biotinylated diphosphine ligands in the study of the hydrogenation of **1** with Rh(biotin)–avidin and –streptavidin hybrids.

pH conditions, while Sav hybrids are better catalysts in acidic media; and (iv) enantioselectivity is fostered via site-directed mutagenesis of the S112 position of Sav, in this study yielding up to 94–96% *ee* for the S112G mutant in combination with a short rhodium–biotin cofactor.

Subsequent studies using the Rh–biotin cofactors in combination with WT and glycosylated avidin and WT Sav and variants with mutations at P64, V47, K80, and S112 allowed the same group to further establish the metal-protein cooperativity in achieving enantioselectivity in artificial metalloenzymes, since mutations closest to the hybridization site resulted in larger variations of the enantiomeric product excess. Quantitative hydrogenation of **1** was achieved with up to 96% *ee* (*R*) with the mutant Sav S112G in combination with a functionalized biotin cofactor with an achiral diphosphine chelate [8].

Along with activity and product enantioselectivity, substrate selectivity can also be promoted by the second coordination sphere in artificial metalloenzymes. By carrying out the hydrogenation of **1** in the presence of its phenyl-substituted derivative acetamidocinnamic acid (**5**), the comparison between terminal and internal olefin hydrogenation was examined in a competitive manner (Figure 6.5) [9]. Several of the combinations of one of the 20 possible Sav S112X mutants with one of 18 Rh(diphosphine-biotin) cofactors showed substrate selectivity, with higher conversions of the smaller substrate **1**. The extent of chemoselectivity could rise to up to quantitative hydrogenation of **1**, while the conversion of **5** reached a mere 10%. Several metalloprotein hybrids resulted in good enantioselective catalysts for both substrates to give hydrogenation products with the same absolute configuration, while specific combinations of the Sav mutants with a rhodium cofactor generally resulted in higher product *ee*'s for substrate **1**. It was also noted that while most conversion differences could be rationalized through sterically demanding interactions, mutations that introduced amino acids with coordinating properties, such as Asp and His, showed largely hampered catalytic activities.

To extend the variability of biotinylated diphosphine ligands screened, Ward and coworkers have also made use of the protected enantiopure amino acids *Boc*-proline and *Boc*-phenylalanine for the construction of ligands bearing different linkers. These were also expected to produce a more rigid

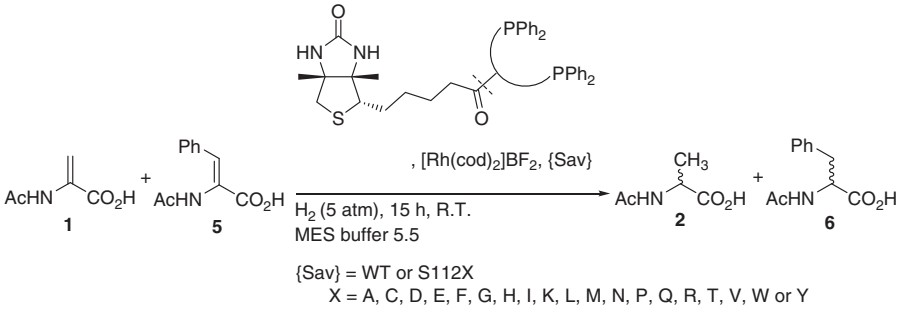

Figure 6.5 Competitive hydrogenation of **1** and **5** by different combinations of Sav mutants and Rh–biotin complexes.

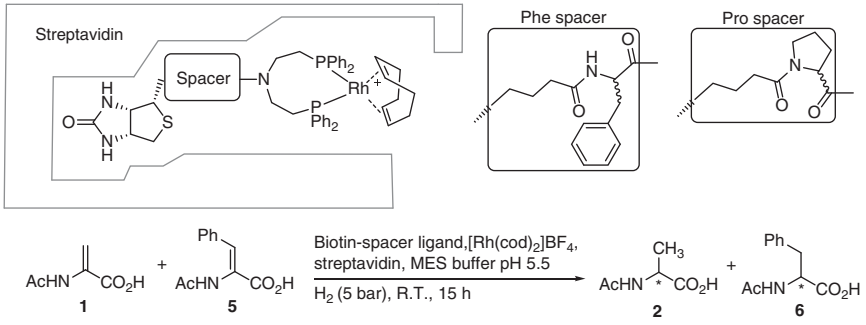

Figure 6.6 Introduction of chiral amino acid spacers in biotin–Sav artificial metalloenzymes.

metalloprotein hybrid and to reach higher *ee*'s by the Rh–protein catalyst. The metalloenzymes constructed with (*R*)- or (*S*)-phenylalanine linkers lead to enantioenriched products from the hydrogenation of compounds **1** and **5**, with small differences in the absolute *ee* of products (66–73% *ee*'s). However, the metalloenzymes with proline linkers led to large differences in the absolute *ee* values, that is, 23% *ee* by the (*S*)-proline and 91% *ee* by the (*R*)-proline, showing again that spatial fixation of the Rh cofactor also depends on the interaction between the cofactor and the protein host. Moreover, the spacer-protein compatibility found in the (*R*)-proline-based metalloenzyme allowed for reproducibility of the catalytic yields and selectivities on hydrogenating **1** and **5** in media with different solvent ratios, where dimethyl sulfoxide (DMSO) as additive in water can be used in as high as 45% volume (Figure 6.6) [10, 11].

One of the desired features of artificial metalloenzymes is the possibility to facilitate organic reactions in aqueous conditions, owing to the intrinsic stability of most proteins in this medium. The metal(biotin)–avidin hybrids can indeed enhance the reactivity of the otherwise unsupported transition metal catalyst in water, thus affording *protein-accelerated catalysis*, since in an aqueous reaction media the metalloenzyme furnished with a hydrophobic metallocatalyst promotes the concentration of hydrophobic substrates in the artificial environment at the active site. Due to the tetrameric quaternary structure of avidin and streptavidin, a peak of acceleration was found when the metal/protein ratio is equal to four [12, 13].

Formally speaking, enantioselectivity optimization by Darwinian mutagenesis supposes mutations of amino acids of random nature and positions in the host protein, followed by a next generation of mutants derived from the most enantioselective resulting hits of the previous round. As introduced earlier, Reetz and coworkers performed a true directed evolution study through full mutagenesis of "close" and "distal" amino acid positions of streptavidin expressed in *Escherichia coli*. Although their computational modeling, positioning the original Whitesides Rh(biotin) cofactor in the protein using the X-ray crystal structure of the biotin–streptavidin adduct, allowed for the identification of amino acids close to the rhodium active site, distal amino acid positions are also important to study since their mutation can derive in overall structural changes of the protein. Five generations of Sav mutants showed a maximum

optimization of Rh(biotin)-Sav after only three mutation rounds, resulting in the hydrogenation of methyl acetamidoacrylate in 65% *ee* by the Asn49Val mutant. This study also showed that not only mutation at the Ser112 position can lead to optimization of the enantioselectivity but that mutations of other positions close to the activity site can do so as well. Only a limited number of mutations in the distal positions resulted in reproducible enantioselective hybrids due to the loss of protein solubility in many of these hybrids [5].

6.2.2 Transfer Hydrogenation of Ketones

The successful approach in olefin hydrogenation using metal–biotin–(strept) avidin systems has initiated the exploration of other catalytic reactions using this conceptual approach in artificial metalloenzymes. In this regard, the Ward group has achieved significant insight in transfer hydrogenation reactions by extending the nature of the biotinylated metal complex to metal(diamine)(arene) systems using ruthenium, rhodium, and iridium, where biotin is furnished with either the diamine or the arene ligand and the counterpart ligand is bound to the metal only.

The different reaction variables – namely, the nature of the metal center and the ligands, the mode of coordination, the protein host (avidin, Sav, and their mutants, including sequenced mutations), the substrate to be reduced, and the hydride source – have been addressed in a sequential approach (Figure 6.7) [14]. At first, when using diamine-functionalized biotin coordinated to ruthenium for

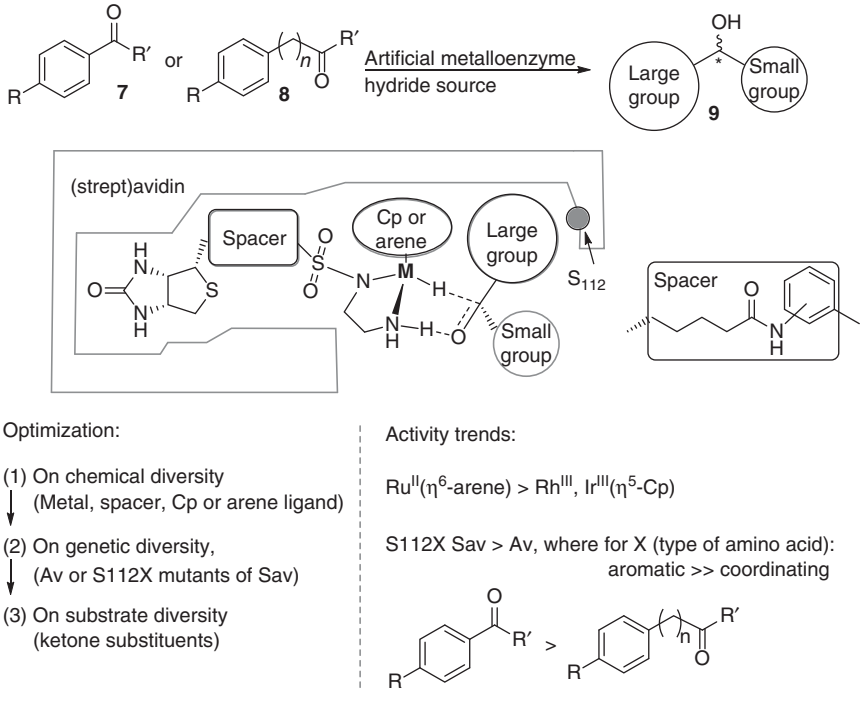

Figure 6.7 Multivariable optimization of artificial transfer hydrogenases (ATHases).

the transfer hydrogenation of *p*-substituted acetophenones (**7**), sodium formate at low concentrations (0.5 M) in the presence of a borate pH buffer was found as the best hydride source; higher concentrations or more aggressive hydrides resulted in protein denaturation. The reaction also performed better with Sav as host protein than avidin, and while the unsupported catalyst gave racemic phenylethanol in the transfer hydrogenation of acetophenone, the artificial metalloenzymes did show enantioselectivity. In the case of Sav hybrids, product enantiomers of opposite configuration result from the choice of benzene or *p*-cymene as the η^6-arene ligand. Point mutations showed that variations at the S112 position stir the extent of conversion, while mutation of the more distant position P64 influenced the enantioselectivity. These discoveries led to the optimization of the system with the combined mutations P64G-S112G to give 92% conversion of acetophenone in 94% *ee* (*R*) [15]. Along with this chemo-genetic study, substrate selectivity was investigated by screening the transfer hydrogenation of acetophenones (**7**) and β- and γ-aryl ketones (**8**) through a range of substituents on the aromatic ring, hence allowing to relate enantiomeric product excess outcome to the difference in size between the substituents of the ketone starting material. General observations in these studies were that (i) the ruthenium hybrids outperformed the rhodium and iridium counterparts, (ii) Sav S112 mutants with amino acids with metal-coordinating features block the catalytic activity, and (iii) mutants with amino acids with aromatic residues afford the highest enantioselectivities toward the (*R*)-product and those with cationic amino acids for the (*S*)-product. With regard to substrate selectivity, similar performance of the hybrids was found with all *p*-substituted acetophenones and with *ee*'s up to 97%; however, enantioselectivity was achieved to a lower extent with nonaromatic ketones [16].

As part of the chemo-genetic optimization of the transfer hydrogenations, computer-generated docking models of the semisynthetic hybrids were generated in order to investigate the chemical environment around the metal center, that is, the metal to amino acid residue distances, and to facilitate the targeting of amino acids for mutation [16]. Shortly after, X-ray analysis of the structure of one of the best (*S*)-selective metalloenzymes, based on the Sav S112K mutant, confirmed that the anchoring of the Ru(biotin) cofactor did not lead to important changes in the structure of the host protein compared with the structure of unmodified host. The crystal structure also allowed for the identification of residues K121 and L124 as additional targets for sequential genetic optimization of the metalloenzymes. With *ee*'s up to 96%, the nature of the arene ligand was also found critical for the enantioselective behavior of the hybrid in the hydrogenation of aromatic ketones. For example, in the case of the S112A-K121N double mutant, the choice of benzene or *p*-cymene again results in opposite product configuration albeit at equivalent substrate conversion [17]. Later on, inclusion of mutations at residues T114, T115, and E116 in this study led to up to 98% *ee* with the P64G-L124V mutant [18]. Important to note is that the Ru(biotin) cofactor itself is chiral at the ruthenium center and that the unsupported and enantiopure (*S*)-Ru(biotin) catalyst is able to afford slight (*S*)-enantioselectivity by itself, thus showing again the enantioselective enhancement by protein hybridization with small molecule catalysts.

6.2.3 Transfer Hydrogenation of Imines

Another interesting class of prochiral substrates for transfer hydrogenation reactions are cyclic imines, in part due to the potential production of tetrahydroisoquinolines, which represent the largest family of naturally occurring alkaloids. Over the last years, Ward and coworkers have focused on the transfer hydrogenation of cyclic imine **10** and its variants as precursors for salsolidines such as **11** (Figure 6.8). Artificial metalloenzymes based on metal(biotin-diamine)(η^n-arene) catalysts and Sav (and its S112 point mutants) afford higher turnover numbers of up to 4000 for this reaction, at pH 6.5 and at milder conditions, than for the transfer hydrogenation of acetophenone. The *ee* of **11** increases upon decrease of the reaction temperature from 55 to only 5 °C, at the cost of extending the reaction times from a few hours to 1–2 days for quantitative yields, yet without the need of degasification of the media prior to the reaction. Interestingly, the hybrids retain their activity under these conditions for as long as 96 h. The iridium catalysts performed better than the rhodium ones in the transfer hydrogenation of the imine. Interestingly, in spite of the tetrameric structure of Sav, the best enantioselectivity was found with less than 4 equivalents of metallic cofactor per protein host, suggesting that an empty binding site adjacent to an occupied site might be favorable for the metal-protein structure. Regarding the role of S112 mutants, the observations are similar to those on the hydrogenation of acetophenone, with cationic amino acids facilitating the formation of the (*S*)-product and the smallest amino acids that of the (*R*)-product [19].

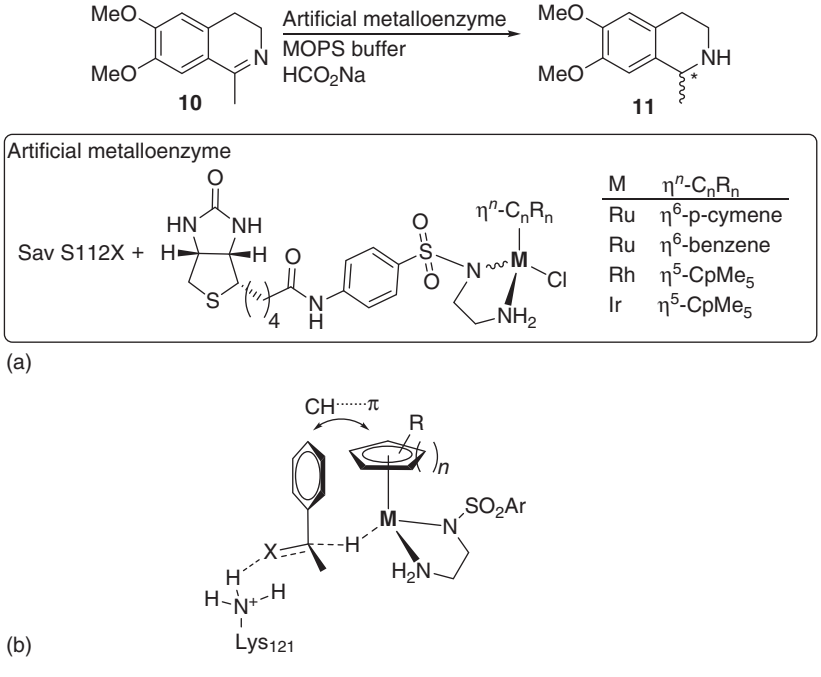

Figure 6.8 Transfer hydrogenation of imine **10** to form salsolidine **11** (a) and the general proposed mechanism for the transfer hydrogenation of ketones and imines (b).

X-ray analysis of the structure of Sav mutant S112A holding biotin with the diamine ligand, yet without a metallic center, allowed for modeling of the metalloenzyme with Ir(η^5-Cp) center and thus the prediction of the steric environment around the active site. The authors proposed a mechanism common for the hydrogenation of the imine and acetophenone substrates, where iridium transfers a hydride to the prochiral atom in the substrate in a non-concerted fashion, stabilized by lysine residue K121 (Figure 6.8b). Mutations at this lysine residue support the proposed mechanism [19].

In view of the phenomenon of protein-accelerated catalysis (see previous text [12]), it was hypothesized that a further increase in the hydrophobicity of the second coordination sphere of the metalloenzyme would lead to a further enhancement in catalytic rate. Kinetic analysis showed that the system obeys Michaelis–Menten kinetics, where the optimized R84A-S112A-K121A Sav mutant used as metalloenzyme scaffold with the [Ir(biotin-diamine)($CpMe_5$)(H)] cofactor (Figure 6.8b) showed a successful 40-fold increase of k_{cat} (37.6 min^{-1}) and eightfold increase in K_M (29.3 mM) in the imine transfer hydrogenation of 1-methyl-3,4-dihydroisoquinoline in comparison with the metalloenzyme with WT Sav [20].

Since a specific amino acid within an artificial metalloenzyme scaffold can affect its catalytic activity and selectivity, it would in principle be possible to design a protein host such that this amino acid is not only part of the second coordination sphere but also acts as a ligand of the metallic cofactor. The ATHases that have been described earlier, based on embedded biotinylated metallic catalysts in (strept)avidin, rely on the functionalization of biotin with a coordinating diamine and an additional "nonsupported" η^5 or η^6 ligand. Their computational and crystal data on Sav-metal hybrids allowed the Ward group to design histidine-containing Sav mutants and to combine these with metal(biotin-η^6-arene) or metal(biotin-η^5-Cp) cofactors, leading to the dual anchoring of rhodium and iridium catalysts in the host protein [21]. The discovery of stabilization in the catalytic transition state by *N*-coordination of the imidazole fragment of residue K121 of Sav, along with the well-known catalytic steering effect of the S112 residue, suggests the mutation of these two amino acids for coordination with the metallic precatalyst. Indeed, Rh(biotin-$CpMe_5$) variants K121H and S112H do not only afford dual anchoring hybridization but also result in pseudo-mirror environments in the metalloproteins, which upon reaction with substrate **10** catalyze the enantioselective hydrogenation toward salsolidine **11** with opposite absolute configuration (Figure 6.9) [21, 22].

Computational docking studies supported the possibility of such dual anchoring of the metallic cofactor to streptavidin through the biotin-Cp ligand and the histidine residues [21]. Such rationalization leads to the proposal that the previous studies on imine reduction with biotin-diamine ligands undergo dual anchoring as well when the histidine or alanine residues were genetically introduced in the same positions of Sav (S112 and K121). This hypothesis was supported once again with computational docking studies of the Ir(biotin-diamine) cofactors in S112A and S112K Sav mutants [23].

In a slightly different approach with Ir(biotin-Cp) cofactors in Sav, it was also discovered that diamine ligands, unlinked to the biotin fragment, can be used

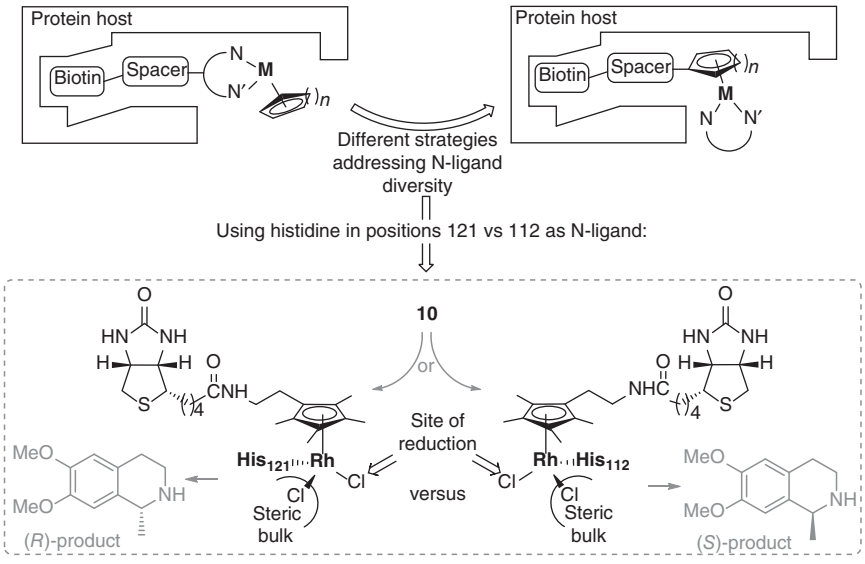

Figure 6.9 Anchoring of Rh catalysts in Sav via dual anchoring of biotinylated η^5-Cp ligands. By using coordinating His residues in different positions of Sav "pseudo-mirror image" catalysts are produced.

to promote modest enantioselectivity in the catalytic reaction when either part of the protein host or as an added small molecular ligand. By using WT Sav in combination with Rh or Ir(biotin-Cp) and a series of diamines such as bipyridine, bisoxazoline, and amino acids with chelating features, full substrate conversion could be achieved with *ee*'s up to 67% [22].

6.2.4 ATHases in Cascade Reactions

The compartmentalization of transition metal catalysts in the cavity of a protein host is an additional feature of metal(biotin)–(strept)avidin hybrids. Hollmann and coworkers reported on catalytic cascade reactions performed by an iridium-based artificial metalloenzyme, a formal ATHase, for the asymmetric transfer hydrogenation of the cyclic imine 1-methyl-3,4-dihydroisoquinoline (**12**) using sodium formate as the hydride source. In the cascade the ATHase is used in combination with other two enzymes in an aqueous reaction mixture: a monoamine oxidases (MAO-N from *Aspergillus niger*) that dehydrogenates (*S*)-amines to the imine by taking O_2 to H_2O_2 and horseradish peroxidase (HRP) that degrades H_2O_2 to water while oxidizing scoleptin (Figure 6.10a). The latter reaction was used as a tool for the colorimetric assay of the cascade reactions, in which the red dye scoleptin decolorates upon oxidation [24]. In the cascade reaction, the ATHase shows a modest enantioselectivity toward (*R*)-amine **13**; the residual (*S*)-product is taken up by MAO-N to regenerate imine **12**. Resembling a dynamic kinetic resolution profile, the orthogonal activity of the ATHase and MAO-N in this way produces enantiopure (*R*)-product **13**. Prior binding of the Ir–biotin cofactor in the pocket of Sav protects its metallic center

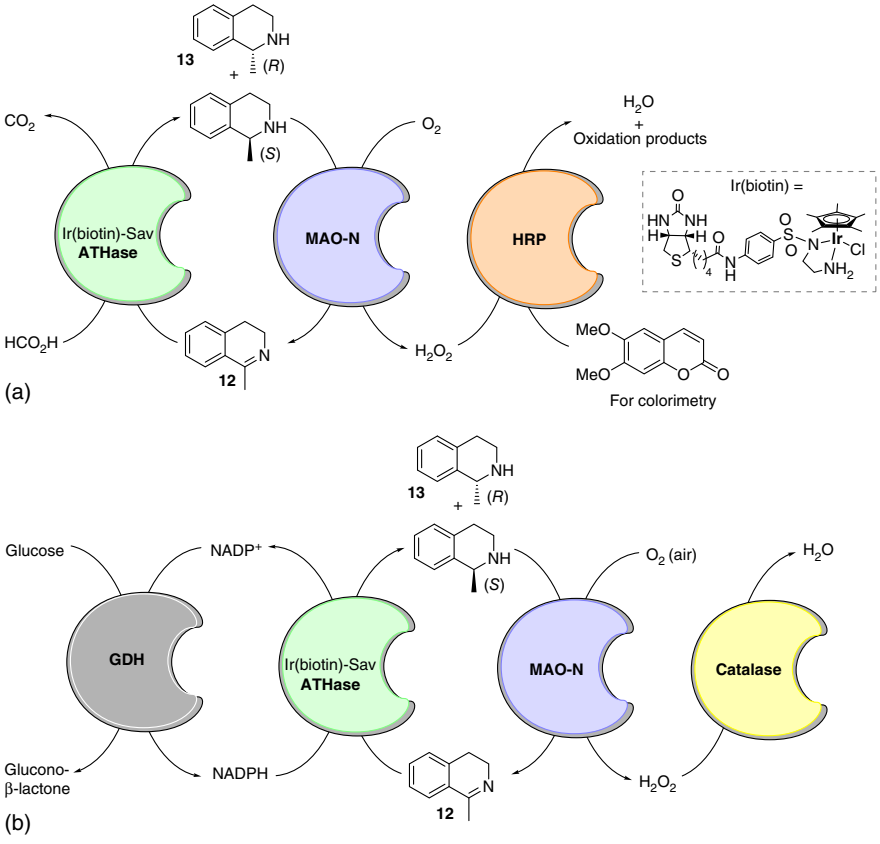

Figure 6.10 Multienzymatic cascade reactions for the transfer hydrogenation of imine **12** toward amine **13** by accumulation of the (*R*)-product using formic acid/formate (a) or glucose (b) as hydride source.

against the oxidizing properties of the other enzymes used in the cascade. The catalytic cascade was also applied in other reactions where the ATHase reduces 2-substituted dihydropyrroles and tetrahydropiperidine [25].

Reductases in biological organisms make use of NAD(P)H as the hydride source. Certainly, the readiness of NAD(P)H in the cell requires a sacrificial energetic – or accumulated – molecule, for instance, glucose, which is converted by an orthogonally coupled enzyme to the reductase. In the spirit of a sound homologation of ATHases with the cell-type enzymatic machinery, NAD(P)H was used as hydride source for Ir(biotin)–streptavidin hybrids in the transfer hydrogenation of imine **12**. The Ir–Sav hybrids were coupled with a glucose dehydrogenase (GDH), a monoamine oxidase (MAO), and a catalase in a cascade enzymatic array for the quantitative enantioselective production of amine **13** in >99% *ee* (*R*) (Figure 6.10b) [26].

Besides asserting that the embedment of metallocatalysts in a protein scaffold allows the use of metalloenzymes in the presence of other active biocatalysts, the use of different enzymes in a cascade fashion is an important step to the

foreseen application of ATHs in cell systems. Moreover, the use of a naturally occurring energetic substrate in the cascade (glucose) in the production of the desired product and water as a by-product is an elegant demonstration of transition metal catalysis and their incorporation in *in vivo*-like transformations.

6.3 Artificial Enzymes with Covalent Metalloprotein Constitution

A different strategy for the development of metalloprotein hybrids is the covalent conjugation of the metallic and the protein components, most commonly via the nucleophilic addition or Michael-type addition of an amino acid residue like cysteine, lysine, or serine to a metallic complex furnished with an electrophilic moiety on one of its ligands. Studies that have applied this hybridization strategy to the development of artificial metalloenzymes for catalytic hydrogenation and transfer hydrogenation reactions have focused on the mono-anchoring of the chemocatalyst to a single, often directed, site of the protein host, for instance, the active site thiol group of cysteine proteases or the active site hydroxyl group of serine hydrolases. One of the strong features of this strategy is the robustness of the resulting metalloprotein hybrid, which can preserve the connectivity between the organometallic fragment and the protein due to the covalent constitution even when a degree of denaturation of the protein scaffold occurs. This feature may be relevant to the catalytic performance of the hybrid and may also facilitate certain treatments and analysis of the hybrid, that is, by means of dialysis or mass spectrometry. When the anchoring addresses the active site of a natural enzyme, as mentioned earlier, the original activity of the enzyme host is lost. Accordingly, the protein scaffold is almost exclusively used as a (chiral) second coordination sphere to the metal complex; nevertheless, protein-induced accelerated catalysis, or dual anchoring, as introduced earlier in this chapter, may also occur. Similar to the biotin–avidin constructs, the incorporated metallocatalyst in covalent hybrids is positioned within a naturally hindered and chemically selective environment, which is a promising feature for a first approximation in bringing about (selective) catalytic properties of the metallocenter.

6.3.1 Papain and Photoactive Yellow Protein

Pioneering work on covalent metalloprotein hybrids was reported by the group of Reetz, with the development of a rhodium(2,2′-dipyridylamine) complex furnished with a maleimide group on the ligand backbone (Figure 6.11a). Maleimides are known electrophiles that can undergo a Michael addition with cysteine residues in proteins, which enabled the Reetz group to anchor the rhodium complex in the active pocket of papain, a cysteine protease that breaks down peptides mainly between hydrophobic amino acid residues. The hybridization inhibited the natural activity of the enzyme and transformed it into a hydrogenase with a preliminary *ee* in catalysis of 10%; the authors, however, did not report details on the catalytic reaction [27].

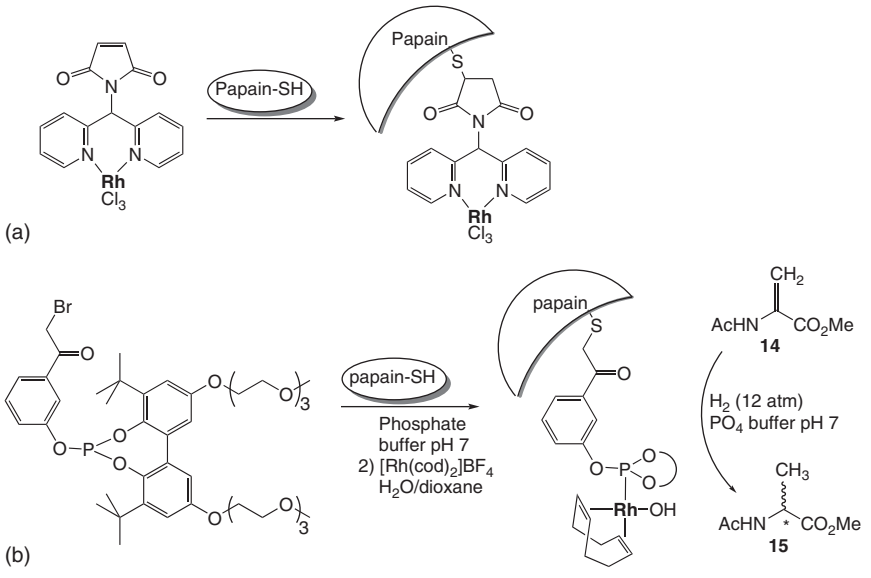

Figure 6.11 Reetz (a) and de Vries (b) approaches for the covalent hybridization of papain with rhodium catalysts for hydrogenation.

De Vries and coworkers treated papain with a rhodium complex derived from a monodentate triphenyl phosphite with a large cone angle and tert-butyl groups on the aromatic rings. The ligand was tethered to an electrophilic bromoacetyl group for the alkylation of papain at the reactive cysteine residue (Figure 6.11b). The resulting hybrid was tested in the catalytic hydrogenation of the hydrophobic methyl acetamidoacrylate **14** to alaninate **15**, for which a complete selectivity for the hydrogenation of the olefin moiety was found, with full conversion of the substrate at 15 h with as low as 0.125 mol% of catalyst loading, albeit without any observable product enantioselectivity [28]. Moreover, the hybrid was successfully characterized by mass spectrometry thanks to its covalent constitution. These early studies by Reetz and de Vries provided a proof of concept for the covalent construction of artificial metalloenzymes and at the same time suggested the need for a more advanced design of the protein host since the papain scaffold provided insufficient burying of the transition metal fragment in order to generate a stereodirecting second coordination sphere.

Salmain and workers performed the alkylation of papain with metallic complexes using both maleimide- and haloacetamide-functionalized ligands. They generated a library of cofactors by tethering either functional group with a short carbon chain to [RuCl(bipyridine)(η^6-arene)]Cl [29] and [RhCl(bipyridine)(η^5-Cp)]Cl moieties for the construction of covalent papain hybrids (Figure 6.12 top and middle) [30]. In the transfer hydrogenation of NAD^+ to NADH with formate as the hydride donor, the Rh^{III} catalysts turned out be a thousand times faster than the Ru^{II} catalysts, both in the papain-supported and nonsupported form. Interestingly, the unsupported rhodium catalysts, for example, $[Rh(Cp^*)(bpy)(H_2O)]^{2+}$, are known to be deactivated in the presence

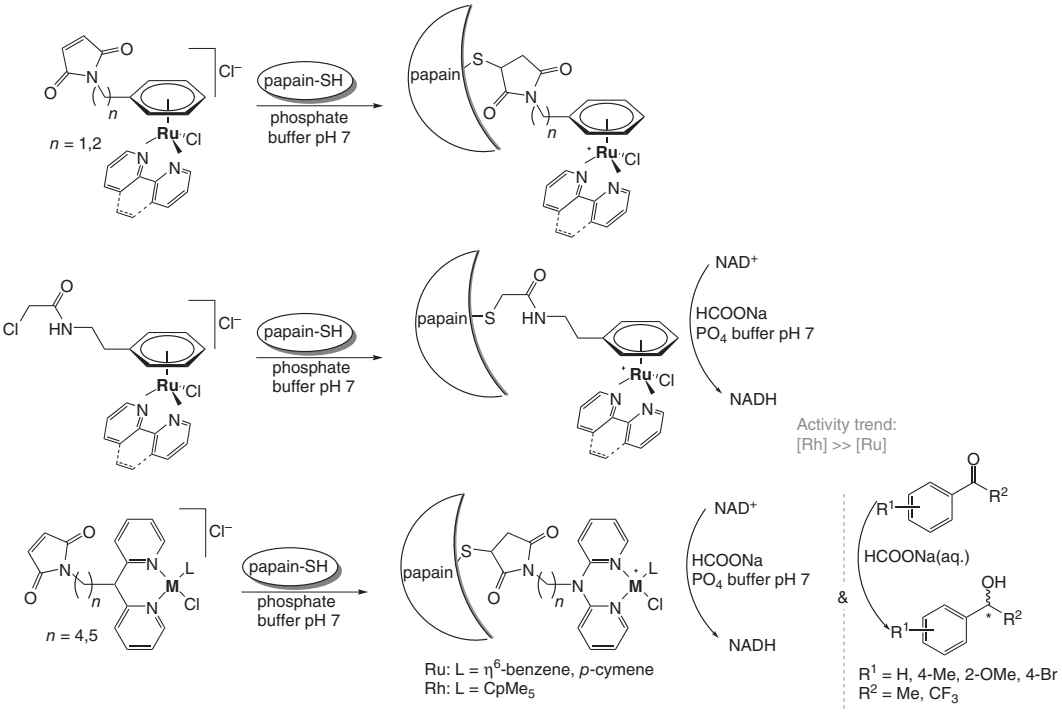

Figure 6.12 Transfer hydrogenation of NAD^+ and acetophenones by Ru and Rh artificial enzymes of papain reported by Salmain.

of proteins in the reaction media as a consequence of nonspecific coordination of the metallic center with peripheral amino acid residues of the protein. The preservation of catalytic activity of the rhodium–papain hybrids therefore demonstrates a beneficial synergistic interaction between the metallic center and the protein scaffold, protecting the metallic center from deleterious nonspecific interactions [30].

The Salmain group also addressed the transfer hydrogenation of acetophenones to study plausible enantioselective features of their covalent artificial metalloenzymes, by comparing ruthenium and rhodium catalysts with 2,2′-dipyridylamine ligands bearing a C3 or C4 tether connecting them to the active cysteine residue of papain, along with different η^5 and η^6 co-ligands such as benzene, *p*-cymene, and Cp* (Figure 6.12 bottom) [31]. Therein, the nature of the Cp or arene co-ligand had a larger influence on the catalytic activity of the metalloenzymes in the hydrogenation of acetophenone and trifluoroacetophenone than the length of the linker, with *p*-cymene as the best co-ligand. High concentration of the reductant favored the reaction extent, with up to 80% yield with 300 equivalents of formate with respect to the substrate. Moreover, dynamical changes in the conformation of the protein could exert an influence on the catalytic properties of the metalloenzymes; due to the pH-dependent stability of the tertiary structure of papain, the metallo-papain hybrids showed a pH-dependent activity profile, with an optimum at pH 3.8. Related to this finding, the need for a buffer is of interest, and, surprisingly, while the presence of buffer (citrate–phosphate) showed no effect on the overall conversion of acetophenone, a higher conversion in the absence of buffer was found for trifluoroacetophenone. With up to quantitative conversion of either ketone under optimized conditions, product enantioselectivities were in the low 0–15% range in favor of the *R*-enantiomer. In a subsequent study dedicated to papain hybrids derived from the rhodium piano stool complexes in this type of catalytic hydrogenation reactions, computational docking studies showed that long distances between the amino acid residues of papain and the metallic fragment resulted in the low catalytic *ee* values [32].

Eppinger and coworkers introduced a dual supramolecular covalent anchoring strategy for the anchoring of $[RhCl_2(PPh_3)(\eta^5\text{-Cp})]$ and $[RuCl_2(PPh_3)(\eta^6\text{-benzene})]$ fragments in papain and two other cysteine proteases, namely, bromelain and cathepsin L. To this end the η^5-Cp or a η^6-benzene ligand was tethered to an epoxide, susceptible to nucleophilic attack by cysteine, and a so-called recognition element. The latter is a relatively short organic fragment for which the host enzyme presents supramolecular affinity through cavity-fragment accommodation along with hydrogen bonding. Placing the epoxide reactive site in the middle of the enzyme inhibitor and thereby placing the metal moiety and the recognition element on opposite sides of the reactive site (Figure 6.13), this inhibition concept helps in fixing the rigidity and directionality of the metallic center in the conjugated hybrid. The resulting artificial metalloenzymes are active in the transfer hydrogenation of substituted acetophenones and trifluoroacetophenone and allow for product *R*/*S* ratios of up to 82 : 18 (64% *ee*) with the rhodium–papain hybrid [33].

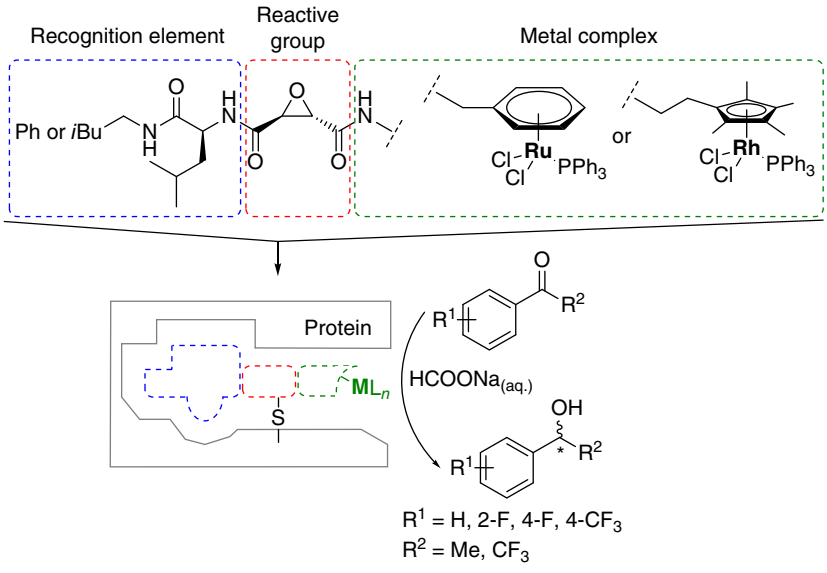

Figure 6.13 Dual (supramolecular covalent) hybridization of papain with Ru and Rh complexes for the enantioselective transfer hydrogenation of acetophenones.

The Kamer group addressed the globular photoactive yellow protein (PYP), which has a more sterically hindered environment around a cysteine residue than papain, to form hybrids with a metallic catalyst in order to promote an efficient second coordination sphere and, accordingly, enantioselective catalysis. Before the alkylation of the cysteine residue, a diphosphine ligand bearing a terminal carboxylic acid chain was reacted with carbonyl diimidazole (CDI), readily forming the carbonyl monoimidazole-functionalized diphosphine. Upon reaction of the metalated derivative with the protein, the imidazole is displaced by the cysteine residue (Figure 6.14) [34]. The constricted environment around Ru and Rh centers in the hybrid prevented the substrate dimethyl itaconate (**16**) to access these. Using an organic co-solvent the substrate could be hydrogenated by the [Rh(diphosphine)(cod)]$^+$ center, yet without showing *ee*'s. Apparently, the organic co-solvent had the side effect of denaturing the tertiary structure of the enzyme, thereby decreasing the capacity to stabilize intermediates with specific enantiomeric conformation.

6.3.2 Serine Proteases

In the last decade, lipases have also received attention as the protein scaffold in covalent active site-directed hybridization methods for the construction of artificial metalloenzymes. Enzymes from the family of serine hydrolases can be inhibited through their reaction with phosphonate esters to become phosphorylated at the active serine residue in an irreversible fashion. Van Koten and Klein Gebbink made use of this concept to covalently tether organometallic motifs bearing a phosphonate ester chain to the active site of lipases using cutinase as

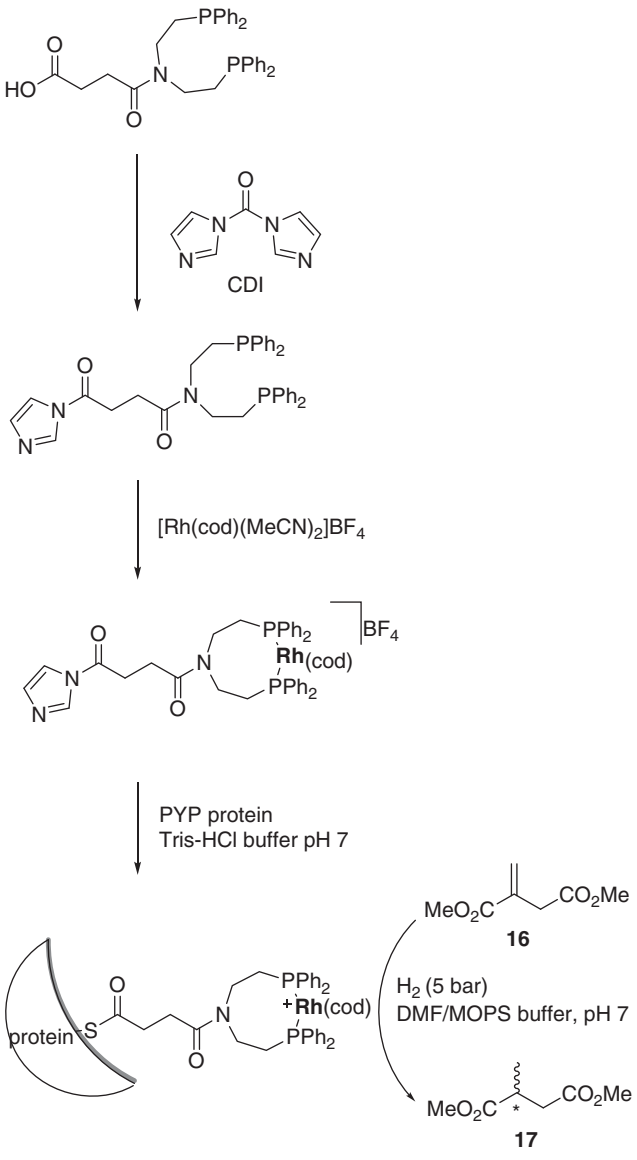

Figure 6.14 Approach for covalent modification of PYP by Kamer.

the key lipase target (Figure 6.15a). In doing so, the natural hydrolytic activity of the host enzyme is lost and replaced by a synthetic organometallic fragment with catalytic or other properties of interest. Characterization of the resulting hybrids was achieved by mass spectrometry under denaturating conditions, lending credit to the covalent anchoring of the organometallic fragment, and in a number of cases by single crystal X-ray diffraction, in all cases providing evidence for selective, single-site hybridization [35]. Early catalytic applications of these lipase-derived hybrids included the Ru(Cp)-catalyzed reversible transfer

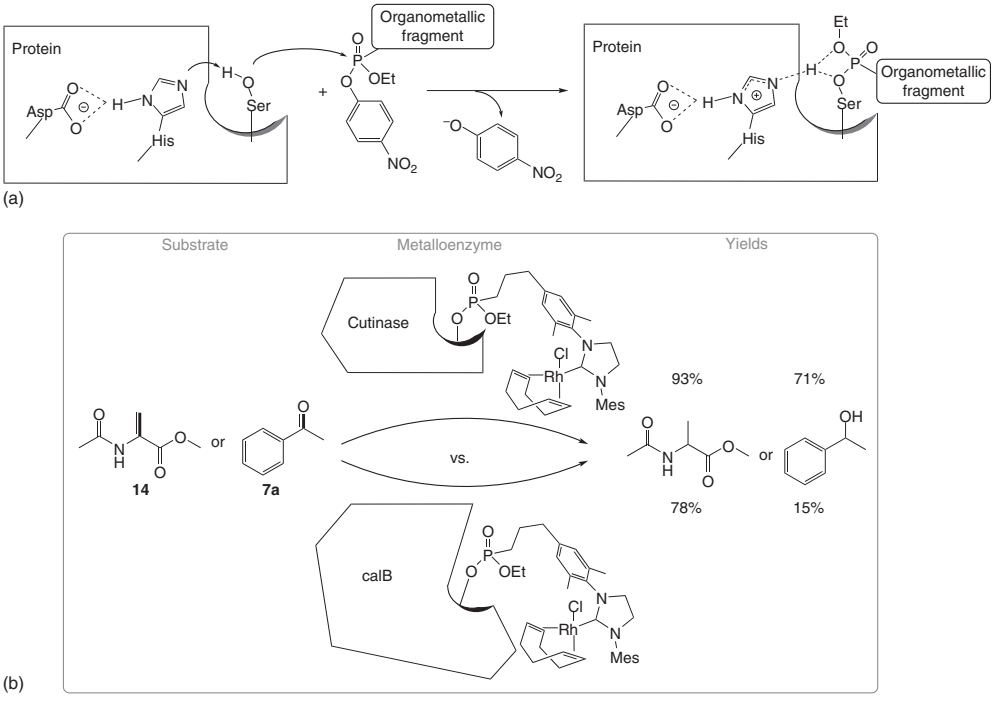

Figure 6.15 (a) General reaction of the covalent, active site-directed hybridization of lipases with organometallic phosphonate inhibitors. (b) Artificial hydrogenation metalloenzymes developed by Klein Gebbink.

dehydrogenation of secondary alcohols, which results in full racemization of the substrate. In a joint study with the Bäckvall group, polymer bead-supported *Candida antarctica* lipase B (CalB) was partially functionalized with the [$RuCl(CO)_2(\eta^5$-Cp-phosphonate)] inhibitor, resulting in a combination of active enzymes, that is, a combination of artificial metalloenzyme-based secondary alcohol racemization and CalB-based enantioselective (*S*)-alcohol acylation. The dual immobilized enzyme system was used in the cascade-type kinetic resolution of secondary alcohols to produce acylated (*S*)-product (>99% *ee*) from racemic 1-phenylethanol, albeit in a modest 38% yield [36].

More recently, the Klein Gebbink group showed that the generation of a second coordination sphere around Rh(NHC) organometallic species incorporated in the active site of the serine hydrolase cutinase enhances the chemoselective activity of the metallic active site in the competitive catalytic hydrogenation of olefins versus ketones (compound **14** vs **7a**) [37]. By placing the organometallic fragment in CalB, known to bear a more constricted active pocket, the enhanced chemoselectivity became more pronounced. At room temperature under hydrogen pressure, the [RhCl(cod)(NHC-phosphonate)] CalB hybrid showed a fivefold preference for the reduction of the olefin versus the ketone in comparison with the cutinase hybrid (Figure 6.15b).

6.3.3 Human Carbonic Anhydrase

Ward and coworkers, inspired by the biotinylated piano stool organometallic compounds used in transfer hydrogenation studies, substituted the biotin motif by an arylsulfonamide functional group, known to bind tightly to the zinc active site present in human carbonic anhydrase II (HCA). After investigation of the hybridization of HCA with ruthenium complexes [38], showing dissociation constants in the micromolar order, they studied iridium-based hybrids (Figure 6.16) to catalyze the TH of salsolidine precursor **10**. First, piano stool iridium complexes bearing arylsulfonamide-functionalized N,N-chelates were tested as catalysts in the absence of HCA host, showing a bipyridine < monopyridine sulfonamide < monopyridine amine trend in catalytic activity. However, once hybridized with HCA, the latter ligand resulted in disappointing activity. Interestingly, the non-fluorinated monopyridine sulfonamide ligand proved active and even a better catalyst than when not hydridized. The iridium complex with the pyridine-sulfonamide chelate could produce salsolidine **11** in 69% yield and 32% *ee* (*S*). By reducing the temperature from 40 to 4 °C, enantioselectivity increased to 68% *ee*, and finally to 70% (*S*) and a yield of 82% when the catalyst loading increased from 1.8 to 9 mol% [39].

6.4 Chemocatalysts Embedded in Protein Motifs

Embedding of catalytically active metallic species in proteins without a specifically targeted site in the protein for the anchoring has been studied as well. This hybridization method offers the possibility of multi-site conjugation, placing

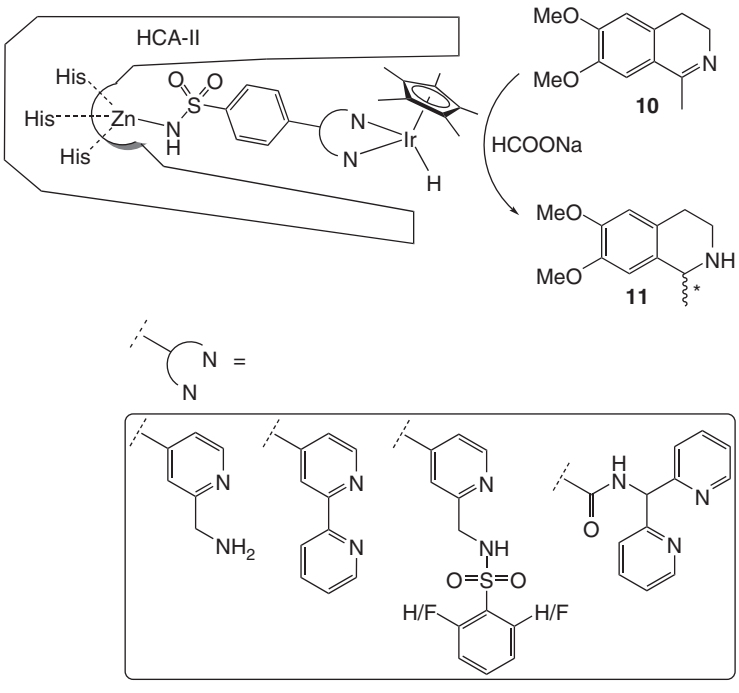

Figure 6.16 Iridium catalysts for the hydrogenation of cyclic imine **10** anchored to HCA II by Zn coordination of tethered diamine ligands.

multiple catalyst units in a protein host, whereas it is difficult to predict the catalytic and, especially, selective properties of the metalloproteins thus prepared. Without the need of a tailored functionality on the catalyst to bind to the protein, this method represents a facilitated strategy for the construction of artificial metalloenzymes. The group of Watanabe has used the iron storage protein ferritin in its metal-free form, that is, apoferritin, to embed palladium nanoclusters by the reaction of tetrachloropalladate in reductive conditions with the protein, resulting in high loading of palladium in the protein's tertiary structure (Figure 6.17) [40]. The resulting hybrid was studied in the olefin hydrogenation of acrylamide

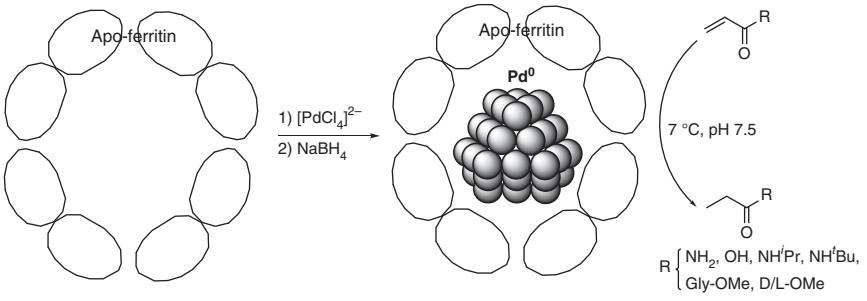

Figure 6.17 Palladium nanoclusters embedded in apoferritin afford a metalloenzyme, which hydrogenates olefins with a TOF that decreases with the size of the substrate.

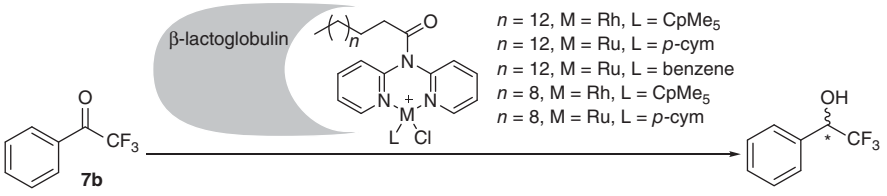

Figure 6.18 The aliphatic affinity of β-lactoglobulin allows the embedding of hydrogenation catalysts of Ru and Rh centers bearing linear hydrocarbon chains.

derivatives indicating a trend in the resulting catalytic turnover frequency, with slower rates for larger substrates, showing that the hybridization brought the size-selective activity to the catalyst.

Salmain and coworkers extended their studies on the transfer hydrogenation of trifluoroacetophenone by ruthenium and rhodium catalysts, originally bound in a covalent mode to papain (see earlier text) [30, 31], through replacement of the cysteine-directed functionalities on the 2,2′-dipyridylamine ligands by aliphatic motifs derived from palmitic and lauric fatty acids (Figure 6.18). The latter were chosen in order to promote conjugation with the protein β-lactoglobulin (β-LG) that is well known to recognize and bind fatty acid derivatives in a 1 : 1 supramolecular fashion with sub-micromolar dissociation constants. Synthetic ligand–β-LG conjugates with and without metal centers were analyzed by circular dichroism (CD), which revealed the successful interaction of the palmitic and lauric fragments with the secondary structure of β-LG and the positioning of the coordination complexes in a chiral environment. The metalloenzymes based on hybrids derived from inhibitors with different aliphatic chains and with Cp, *p*-cymene, or benzene as the additional ligand to either Ru or Rh centers proved to be stable under the reactions conditions required for the transfer hydrogenation of trifluoroacetophenone (**7b**) with formate as hydrogen source for prolonged times of up to 4 days and afforded enantioselectivity in modest *ee*'s between 16 and 26% [41].

6.5 Conclusions

This chapter has highlighted the conceptual development of AHases and ATHases through the (non)covalent combination of a proteinic host and an organometallic catalyst. The broad scope and thorough understanding of catalytic (transfer)hydrogenation reactions in the homogeneous catalysis field have allowed the development of artificial metalloenzymes for these reactions that display, among other aspects, full enantioselectivity as well as inverted enantioselectivity at a relatively early stage of conceptual development. Accordingly, the further development and optimization of AHases and ATHases has embraced the full repertoire of molecular biology tools, including site-directed and random mutation and directed evolution. Further conceptual development of the general field of artificial metalloenzymes is expected to continue to use

catalytic (transfer)hydrogenation reactions as a key enabling tool alongside new molecular biology tools.

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