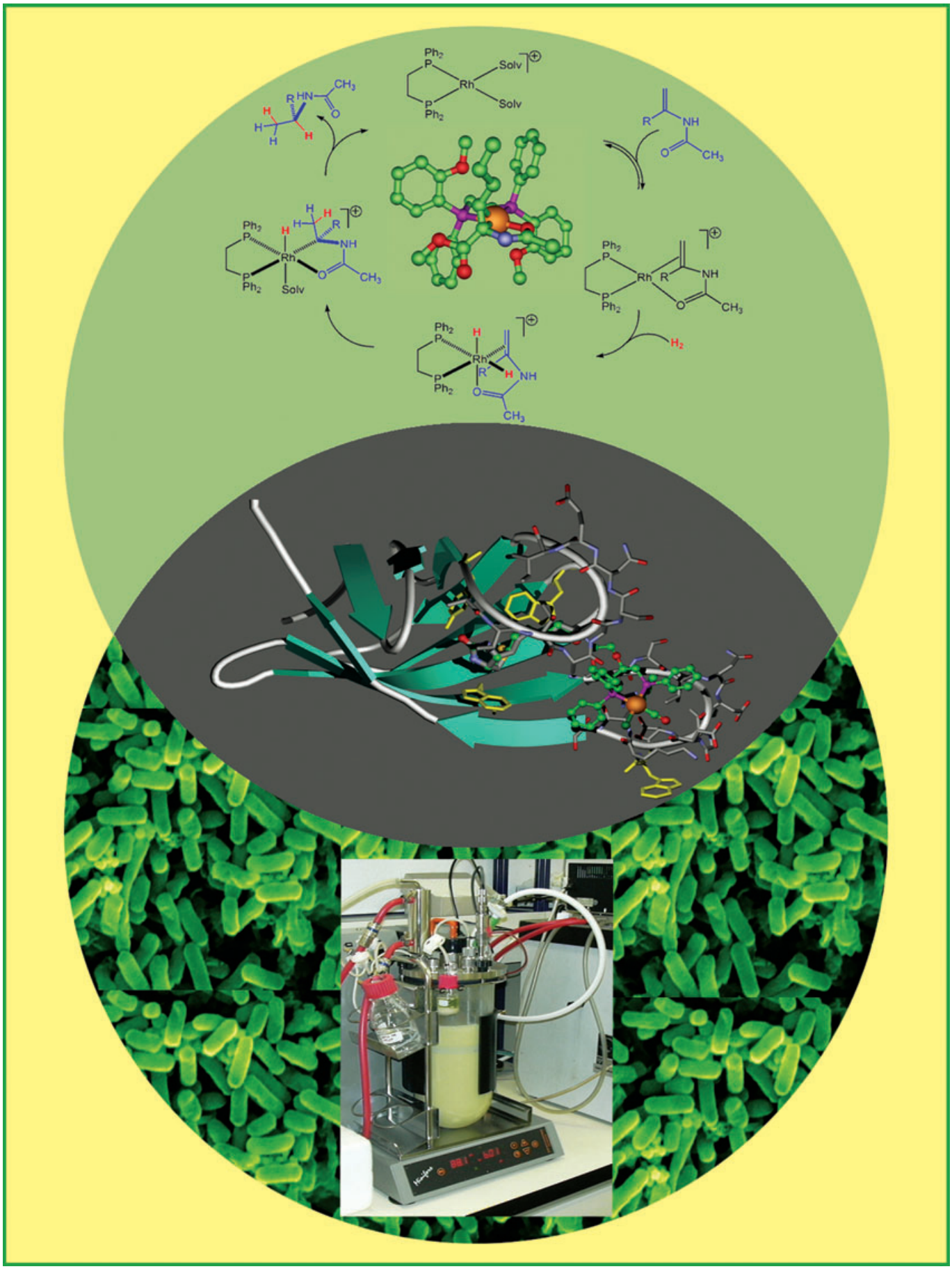
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Artificial Metalloenzymes for Enantioselective Catalysis Based on the Noncovalent Incorporation of Organometallic Moieties in a Host Protein

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Abstract: Enzymatic and homogeneous catalysis offer complementary means to produce enantiopure products. Incorporation of achiral, biotinylated aminodiphosphine–rhodium complexes in (strept)avidin affords enantioselective hydrogenation catalysts. A combined chemogenetic procedure allows the optimization of the activity and the selectivity of such artificial metalloenzymes: the reduction of acetamidoacrylate proceeds to produce *N*-acetamidoalanine in either 96% *ee* (*R*) or 80% *ee* (*S*). In addition to providing a chiral second coordination sphere and, thus, selectivity to the catalyst, the phenomenon of protein-accelerated catalysis (e.g., increased activity) was unraveled. Such artificial metalloenzymes based on the biotin–avidin technology display features that are reminiscent of both homogeneous and of enzymatic catalysis.

Keywords: artificial metalloenzymes · asymmetric catalysis · biotin–avidin technology · chemogenetic optimization · hydrogenation

Introduction

Traditionally, catalysis has been divided in three distinct categories: heterogeneous, homogeneous, and enzymatic catalysis. Although all three disciplines have been successfully used for enantioselective transformations, the last two have found broader applications in the synthesis of high-added-value enantiopure compounds.[1,2]

In the past thirty five years, metal-catalyzed enantioselective transformations have enjoyed significant growth as it was recognized that these are amongst the most efficient ways to produce enantiomerically pure compounds, culminating with the 2001 Nobel Prize in Chemistry awarded to Knowles, Noyori, and Sharpless.[3–5] In recent years, organocatalysis has had an increasing success and impact.[6]

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

To get round the difficulty of predicting the enantioselectivity, combinatorial methodologies have been recently applied to the discovery and development of new enantioselective catalysts.[7,8] These studies have highlighted the fact that many subtle experimental parameters (solvent, counterion, added salts, etc.) often have a significant and unpredictable influence on the enantioselectivity of a reaction. These weak contacts between a catalyst and its "nonbonded" environment are commonly referred to as the second coordination sphere.[9]

In contrast to organometallic catalysts, enzymes exquisitely tailor both the first and second coordination spheres of their active site to afford efficient and selective catalytic systems. This characteristic is very hard to achieve in homogeneous catalysis, in which the steric and electronic control is mostly limited to the first coordination sphere of the metal. It is instructive to note that, in bioinorganic chemistry, very few model systems that faithfully reproduce a metallo-coenzyme's first coordination sphere are as active and as selective as the true metalloenzyme in which both the first and the second coordination sphere have been optimized by evolution.[10]

Biocatalysis offers an attractive alternative to the synthesis of enantiopure products.[2,11,12] In particular, the recent implementation of directed evolution techniques (combined with an efficient screening[13] or selection tool[14]) has overcome some of the inherent limitations of enzymatic catalysis

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(e.g., giving access to both enantiomers of the product), thus significantly expanding the scope of applications.[15–20] A rough comparison of enzymatic and homogeneous catalysis is presented in Table 1.

Table 1. Comparison of typical features of enzymatic and homogeneous catalysis.

	Enzymatic Catalysis	Homogeneous Catalysis
reaction repertoire	small	large
turnover number	large	small
optimization	genetic	chemical
second coordination sphere	well defined	ill defined
substrate scope	small	large
enantiomers	single enantiomer	both enantiomers
reaction medium	mostly aqueous	mostly organic
catalyst recovery	straightforward	difficult

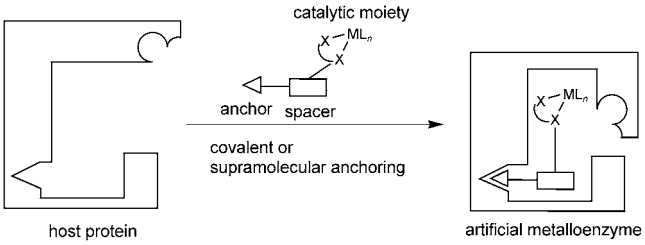
From the considerations outlined in Table 1, it appears that enzymatic and homogeneous catalysis are, in many respects, complementary. A catalyst which would combine the best of both these kingdoms may appear as a perfect catalytic system!

With this goal in mind, artificial metalloenzymes that combine an organometallic moiety embedded in a protein environment have received increasing attention recently.[21] In a biomimetic spirit, both the first and second coordination spheres may be optimized to produce versatile enantioselective catalysts. This novel approach offers several appealing features:

1) The possibility of dissociating the activity (primarily dictated by the organometallic catalyst precursor) from the selectivity (governed by the host protein).
2) The use of orthogonal diversity-generating procedures: molecular biology for the protein optimization as well as parallel synthesis for the organometallic fragment.
3) A novel approach to exploit weak interactions in enantioselective catalysis.

To unambiguously localize the organometallic moiety within the host protein, two complementary approaches can be envisaged (Scheme 1): 1) covalent anchoring and 2) supramolecular anchoring.

1) *Covalent anchoring*: Inspired by the early work of Kaiser,[22] several groups have developed methods to covalently modify proteins by incorporating transition-metal catalysts to yield hybrid catalytic systems with promising properties. For this purpose, a protein with a single accessible reactive amino acid residue (typically a cysteine or a serine residue) is covalently coupled to an organometallic moiety incorporating a complementary reactive functionality.[21,23–25]



Scheme 1. Anchoring of an active catalyst within a host protein affords artificial metalloenzymes for enantioselective catalysis. The host protein displays high affinity for the anchor (triangle); introduction of a spacer (rectangle) and variation of the ligand scaffold allows to chemically optimize the selectivity of the hybrid catalyst. Site-directed mutagenesis allows a genetic optimization of the performance of the artificial metalloenzyme.

2) *Supramolecular anchoring*: To ensure the localization of the organometallic moiety within the host protein, a very strong noncovalent guest⊂host (i.e., an inhibitor⊂protein) couple should be selected. Since no chemical coupling step is required upon addition of the catalyst precursor (which contains the inhibitor acting as anchor) to the protein, the integrity of the organometallic species is warranted.[26] Ideally, the host protein should possess a deep binding pocket capable of accommodating an organometallic moiety. Furthermore, the affinity of the inhibitor for the protein should not be too strongly affected upon coupling of the inhibitor to a large organometallic moiety.

For supramolecular-anchoring purposes, the biotin–avidin couple fulfils all of the above requirements. The association constant of biotin for avidin is the highest known in nature for a noncovalent interaction ($K_a \approx 10^{14}\,\text{M}^{-1}$).[27] In addition to the six hydrogen-bonding contacts between the protein and the biotin's bicyclic urea framework, the binding pocket possesses four or five (for streptavidin and avidin, respectively) aromatic residues that make up a deep hydrophobic box, Figure 1[28,29] The biotin–avidin technology relies primarily on the fact that derivatization of the valeric acid side chain of biotin does not reduce significantly the affinity of biotin for (strept)avidin (hereafter (strept)avidin refers to either avidin or streptavidin).[27] In the biotin–avidin technology, however, a long spacer (C_5 or longer) is usually introduced between the biotin anchor and the conjugated probe. Additional appealing aspects of the biotin–(strept)-avidin system include: 1) both proteins have been expressed in various organisms, 2) the proteins are very robust, and 3) the proteins are easy to purify by affinity chromatography.

Inspired by Whitesides' and Chan's early reports, we focused on artificial metalloenzymes based on biotin–avidin technology for the hydrogenation of acetamidoacrylic acid.[30,31]

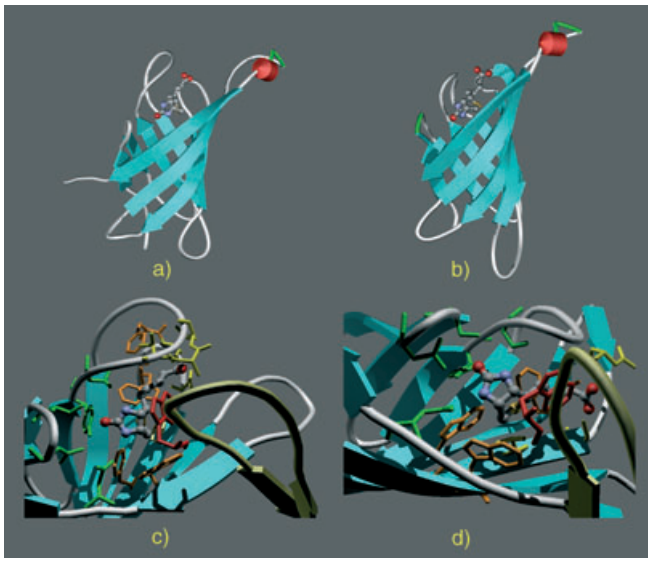
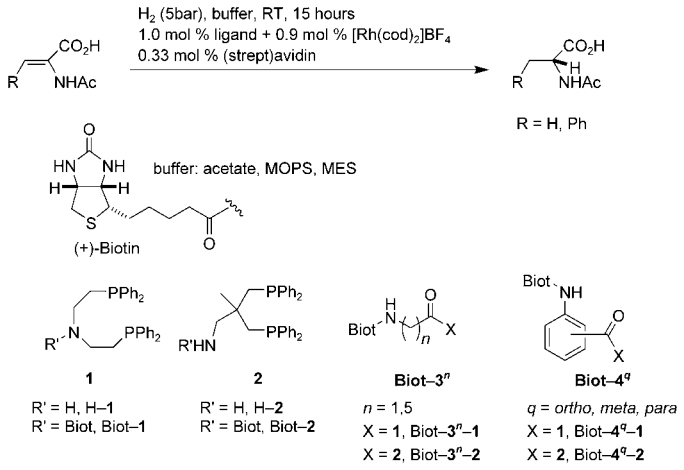


Figure 1. The biotin–(strept)avidin complex as revealed by X-ray crystallography. One monomer of the tetrameric (strept)avidin structure is depicted, emphasizing the eight-stranded β-barrel fold as well as the deep binding pocket for biotin (ball and stick) in a) avidin (pdb refcode: 1avd) and b) streptavidin (pdb refcode: 1swd). Close-up view of a biotin binding pocket in c) avidin and d) streptavidin. Biotin (ball and stick); polar amino acid residues (green and yellow) interacting with biotin's urea functionality (green residues) and with the valeric acid (yellow residues); aromatic residues forming the hydrophobic box are highlighted in orange and red. This last tryptophan residue is supplied by an adjacent monomer (gold loop), providing a lid to the hydrophobic box.

Artificial Metalloenzymes Based on Biotin–Avidin Technology

Enantioselective hydrogenation: Two achiral aminodiphosphine ligand scaffolds **1** and **2** were selected for screening purposes (Scheme 2). Upon coordination, the biotinylated ligand Biot-**1** forms an eight-membered ring, thus conferring significant flexibility to the resulting chelate. In contrast, the biotinylated ligand Biot-**2** is more rigid upon chelation. Six-membered aliphatic-ring diphosphine ligands are known to perform poorly in enantioselective catalysis as they often adopt pseudo-achiral chair conformations.[32,33]



Scheme 2. Operating conditions and ligands used in conjunction with (strept)avidin for the hydrogenation of *N*-acetamidodehydroaminoacids.

The first catalytic experiments were performed with commercial egg-white avidin. The results were disappointing as the enantiomeric excess (*ee*) did not exceed 39% (*S*) when Biot-**1** was used (Scheme 2).[30] As the isoelectic point of avidin is very high (pI = 10.4), we suspected that, at neutral pH, significant amounts of the cationic catalyst precursor $[Rh(Biot\text{-}\mathbf{1})(cod)]^+$ (cod = cyclooctadiene) may not be incorporated within the cationic host protein.

We thus turned to streptavidin, a structurally related protein (30% sequence homology) with a lower isoelectric point (pI = 6.2). To our delight, the hydrogenation of acetamidoacrylate in the presence of $[Rh(Biot\text{-}\mathbf{1})(cod)]^+ \subset$streptavidin (1 mol%) proceeded quantitatively and with good enantioselectivity (94% *ee* (*R*)) (Scheme 2).[34] Combining both acetamidoacrylic acid with acetamidocinnamic acid substrates and the use of $[Rh(Biot\text{-}\mathbf{1})(cod)]^+ \subset$streptavidin (in 0.1 M MES buffer at pH 5.5; MES = 2-morpholinoethanesulfonic acid) yielded (*R*)-acetamidoalanine and (*R*)-acetamidophenylalanine in 94% *ee* (quantitative and 85% conversion, respectively) in both cases.[35] This suggests that the artificial metalloenzyme operates indiscriminately on both substrates, irrespective of their size, reminiscent of homogeneous catalysts.

Introduction of an achiral amino acid spacer between the biotin anchor and the {Rh(diphosphine)} moiety makes it possible to chemically optimize the selectivity of the artificial metalloenzyme. For this purpose, five alkylaminoacids (**3**^{n}, n = 1–5) and three arylaminoacids (**4**^{q}, q = *ortho*-, *meta*- and *para*-) were combined with both ligand scaffolds **1** and **2** to afford a total of eighteen biotinylated ligands (including the ligands Biot-**1** and Biot-**2**, devoid of spacer). Screening revealed that $[Rh(Biot\text{-}\mathbf{3}^1\text{–}\mathbf{2})(cod)]^+ \subset$avidin affords acetamidoalanine in 80% *ee* (*S*). This last result demonstrates that both avidin and streptavidin are suitable host proteins for the creation of artificial metalloenzymes.

The screening revealed that the pH as well as the nature of the buffer have some influence on both the yield (± 10%) and on the enantioselectivity (± 5%). Overall, avidin performs best at neutral pH (0.1 M MOPS buffer; MOPS = 3-(*N*-morpholino)propanesulfonic acid), whereas streptavidin performs best at pH 4.0 (0.1 M acetate buffer), further suggesting that the ionization state of the host protein has little influence on the performance of the artificial metalloenzyme.

Chemogenetic optimization procedure: Having validated the chemical optimization strategy, we next moved to the genetic optimization of selectivity. For this purpose, four streptavidin mutants (S112G, V47G, K80G, and P64G) were expressed in multigram quantities in *E. Coli*,[36] purified, and tested in combination with the eighteen above ligands.[37] In all cases, a loop residue was substituted by a glycine residue, thus imparting conformational freedom to the corresponding loop.

1) Docking experiments suggest that residue S112 (Loop 7,8) lies close to the [Rh(Biot-**1**)]$^+$ moiety upon incorporation in streptavidin.
2) Removing a positively charged lysine residue in the vicinity of the active site (K80, Loop 5,6) is expected to influence to position of the cationic rhodium moiety.
3) The main chain carbonyl group of V47 (Loop 3,4) displays a second coordination sphere contact with biotin: it interacts with the critical S45, which, in turn, interacts with one proton of the urea functionality of biotin.[29]
4) Removing the constraint imposed by a proline (P64, Loop 4,5) residue may be subtly reflected in the remote biotin binding site.

In addition to the streptavidin mutants described above, a recombinant glycosylated form of avidin was expressed in *Pichia pastoris*.[38] Four point mutations, K3E, K9D, R122A, and R124A, as well as an additional E-A-E at its N-terminus yielded an avidin mutant (r-GAvi) with a pI = 5.4. In strong contrast to egg-white avidin, r-GAvi displays a very narrow glycosylation pattern (primarily Man_9-$(GlcNAc)_2$, Man = mannose, GlcNAc = *N*-acetylglucosamine).

The results of the screening experiments with acetamidoacrylate as substrate are summarized using a fingerprint for each matrix element (18 × 7 ligand–protein combinations) in Figure 2:[39] the strawberry color codes for (*S*)-acetamidoalanine and the green color codes for the (*R*)-acetamidoalanine. The intensity of the color is proportional to the conversion. Such a display format allows the qualitative rapid identification of the best ligand–protein combinations, as well as general trends. Inspection of both S112G and V47G vectors reveals that these two mutants are anticorrelated: the Biot-**1**/S112G combination affords (*R*)-acetamidoalanine in 96 % *ee*, whereas the Biot-**1**/V47G matrix element affords (*R*)-acetamidoalanine in only 26 % *ee*. The couples (Biot-**4**^{ortho}–**2**/S112G (57 % *ee* (*S*)) and (Biot-**4**^{ortho}–**2**/V47G (44 % *ee* (*R*)) display the greatest degree of anticorrelation.

The conversions obtained using Biot-**4**^{para}–**2** in combination with streptavidin and its mutants are moderate (50–70 %) in all but one case (K80G, quant. conversion), suggesting that the catalytic moiety is not very accessible with this ligand–spacer combination. Despite a lowered isolectric point and its well-defined glycosylation pattern r-GAvi performs more poorly than WT-Avi.

The above experiments demonstrate that the *selectivity* of artificial metalloenzymes is amenable to a chemogenetic optimization procedure. Next, we analyzed the effect of the protein environment on the *activity* of the hybrid catalyst.

Protein-accelerated catalysis: Both in the areas of homogeneous and heterogeneous catalysis, the concept of ligand acceleration has proven valuable.[40,41] In ligand-accelerated catalysis, the presence of a ligand increases the reaction rate of a catalytic transformation, which proceeds even in the absence of added ligand. The same concept may apply for artificial metalloenzymes as a biotinylated catalyst precursor (e.g. [Rh(Biot-**1**)(cod)]$^+$) is active outside its host protein, producing racemic material (Scheme 3). Assuming that both competing catalytic cycles (within and without (strept)avidin, once all binding sites are saturated) proceed according to the same mechanism, the ratio of the rates $k_{cat\subset prot}/k_{cat}$ can be estimated by incrementally varying the [Rh(Biot-**1**)-(cod)]$^+$/(strept)avidin ratio. For example, in the presence of eight equivalents [Rh(Biot-**1**)-(cod)]$^+$ versus (strept)avidin, four equivalents (producing acetamidoacrylate in 94 % *ee* (*R*) for streptavidin and in 39 % *ee* (*S*) for avidin) are located within the host protein and four equivalents (producing racemic material) are located outside the host protein. Assuming the same rates ($k_{cat\subset prot} = k_{cat}$), the expected *ee* for acetamidoacrylate is (94+0)/2 = 47 % *ee* for streptavidin and (39+0)/2 = 20 % *ee* for avidin. The *ee*'s obtained with eight equivalents biotinylated catalyst are 75 % *ee* and 36 % *ee* with streptavidin and with avidin, respectively. Such an increase of activity of the catalyst within the protein cavity, reflected in the enantiomeric excess of the product, suggests

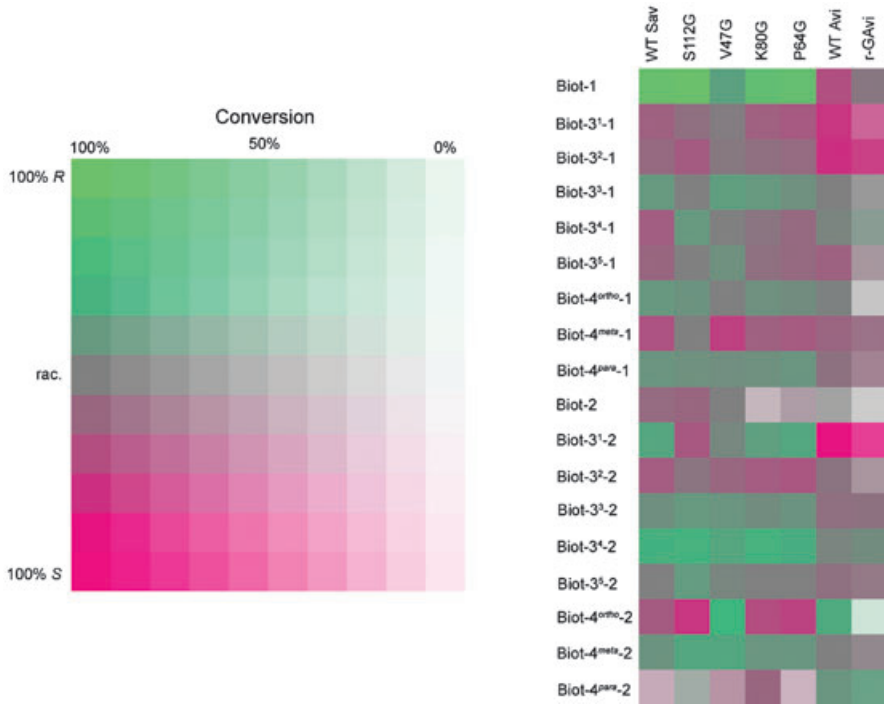
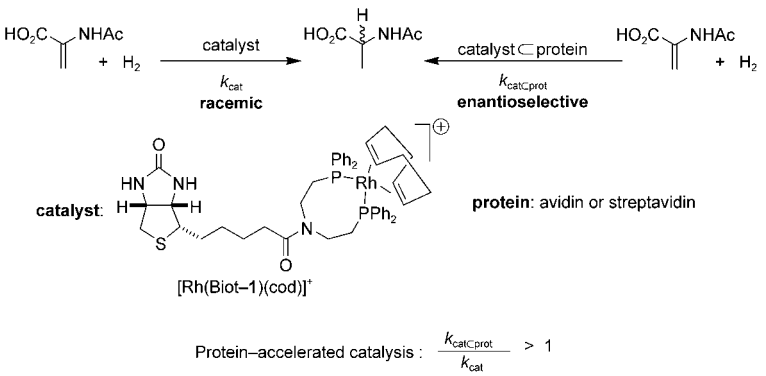


Figure 2. Selectivity array (fingerprint) for the reduction of acetamidoacrylate using artificial metalloenzymes based on the biotin-avidin technology.



Scheme 3. The concept of protein-accelerated catalysis. The protein-embedded catalyst [Rh(Biot-**1**)(cod)]$^+$ ⊂(strept)avidin produces enantioenriched reduction product, while the protein-free catalyst [Rh(Biot-**1**)-(cod)]$^+$ affords racemic product.

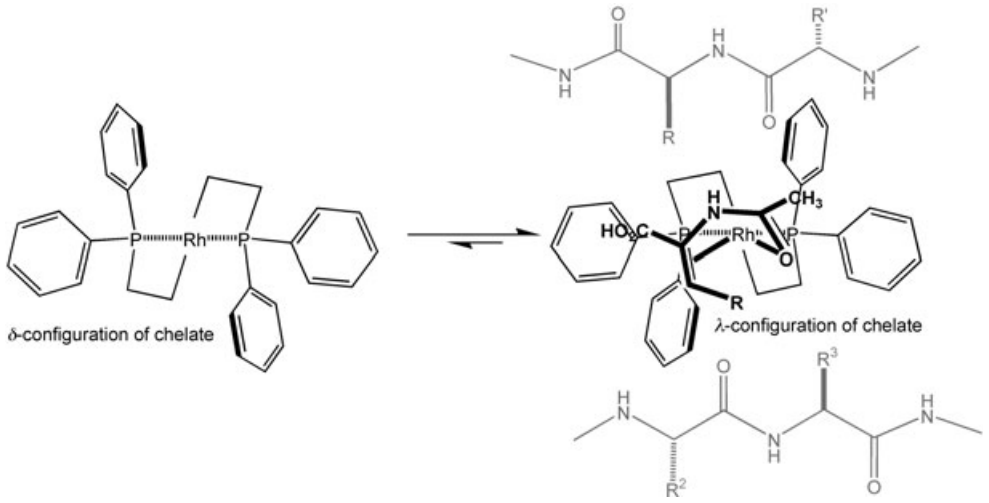
protein-accelerated catalysis.[42] A possible explanation for this phenomenon may be the presence of a hydrophobic binding pocket (Figure 1) leading to an accumulation of the hydrophobic substrate within the host protein. This, in turn, is reflected by an increase in the rate $k_{cat⊂prot}$ versus k_{cat}. In the close future, we plan to carry out a thorough kinetic analysis on such systems. So far, such an analysis has been hampered by the low solubility of the substrates, preventing us from varying their concentration over a wide range to extract the precious k_{cat} and K_M parameters.

Outlook

Confronting the observations outlined herein with Table 1 reveals that the artificial metalloenzyme based on biotin–avidin technology allies features that are reminiscent both of the enzymatic and homogeneous catalysis.

1) The approach broadens the scope of application of enzymes. Incorporation of an active organometallic moiety (which is compatible with aqueous media) in a host protein should ideally allow us to perform reactions catalyzed by artificial metalloenzymes that are unique to the organometallic kingdom.
2) The turnover number of the hybrid catalysts is modest: 1 mol% rhodium is typically used. This is due to traces of oxygen that irreversibly poison the rhodium–diphosphine moiety. The hybrid catalyst can be readily separated from the reaction mixture by size-selective filtration. As the biotinylated catalyst is noncovalently bound, a single denaturation–renaturation cycle restores biotin-binding activity, thus allowing the protein to be recycled.
3) Chemical and genetic methodologies can be combined to optimize both the activity and the selectivity of the artificial metalloenzymes. By using this procedure, both enantiomers of the product can be obtained.
4) This approach allows us to dissociate the activity from the selectivity of the hybrid catalyst. The activity is by-and-large dictated by the biotinylated organometallic fragment, whereas the selectivity is governed by the host protein. We speculate that, upon incorporation in the host protein, the rhodium-bound diphosphine ligand (which exists as a racemic mixture of δ and λ conformers) adopts an enantioenriched configuration that favors the approach of one of the prochiral faces of the substrate, Scheme 4.

Encouraged and stimulated by these findings, we are currently focusing on mechanistic and structural aspects of the



Scheme 4. Upon incorporation in (strept)avidin (gray), the biotinylated chelate (black) adopts an enantioenriched configuration (either λ or δ) that, in turn, determines which prochiral face of the substrate (bold) binds to the rhodium.

hydrogenation reaction. In addition, we are working on C–C bond-forming reactions catalyzed by artificial metalloenzymes.

Acknowledgements

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