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Second Generation Artificial Hydrogenases Based on the Biotin-Avidin Technology: Improving Activity, Stability and Selectivity by Introduction of Enantiopure Amino Acid Spacers

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Abstract: We report on our efforts to create efficient artificial metalloenzymes for the enantioselective hydrogenation of *N*-protected dehydroamino acids using either avidin or streptavidin as host proteins. Introduction of chiral amino acid spacers – phenylalanine or proline – between the biotin anchor and the flexible aminodiphosphine moiety **1**, combined with saturation mutagenesis at position S112X of streptavidin, affords second generation artificial hydrogenases displaying improved organic solvent tolerance, reaction rates (3-fold) and (*S*)-selectivities (up to 95% *ee* for *N*-acetamidoalanine and *N*-acetamidophenylalanine). It is shown that these artificial metalloenzymes follow Michaelis–Menten kinetics with an increased affinity for the substrate and a higher k_{cat} than the protein-free catalyst (compare k_{cat} 3.06 min^{-1} and K_M 7.38 mM for [Rh(COD)**Biot-1**]$^+$ with k_{cat} 12.30 min^{-1} and K_M 4.36 mM for [Rh(COD)**Biot-(*R*)-Pro-1**]$^+$ ⊂ WT Sav). Finally, we present a straightforward protocol using Biotin-Sepharose to immobilize artificial metalloenzymes (>92% *ee* for *N*-acetamidoalanine and *N*-acetamidophenylalanine using [Rh(COD)**Biot-(*R*)-Pro-1**]$^+$ ⊂ Sav S112W).

Keywords: artificial metalloenzymes; biotin-avidin; chemogenetic optimization; enantioselective catalysis; enzyme immobilization; hydrogenation

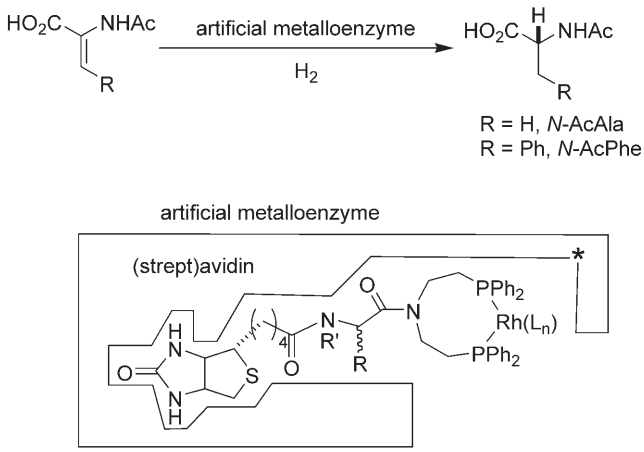
Introduction

Today, the preparation of enantiomerically pure compounds is one of the most actively pursued fields in synthetic chemistry. In this major effort, both homogeneous and enzymatic catalysis occupy a place of choice.[1–3] To complement these well established methods, organocatalysis and artificial metalloenzymes have witnessed a revival in recent years.[4]

The underlying principle of artificial metalloenzymes for enantioselective catalysis is based on the incorporation of an active catalyst within a macromolecular host (protein or oligonucleotide) which provides a chiral environment, responsible for the selectivity.[5–10] To ensure localization of the catalytic moiety within the macromolecular host, covalent, dative and supramolecular anchoring strategies have successfully been exploited to produce enantioselective hybrid catalysts for ester hydrolysis,[11] dihydroxylation,[12] epoxidation,[13,14] sulfoxidation,[15–19] hydrogenation,[20–28] transfer hydrogenation[29,30] and Diels–Alder reactions.[31–33]

Based on Whitesides' early report,[20] several groups have been exploiting the biotin-avidin technology to produce artificial hydrogenases for the enantioselective reduction of *N*-protected dehydroamino acids[21–26,28] as well as the reduction *via* transfer hydrogenation of aromatic ketones.[29,30]

For optimization purposes, our group relies both on chemical and on genetic strategies (i.e., chemogenetic)[5] to yield both (*R*)- and (*S*)-selective hydrogenases. For the proof-of-principle, we focused exclusively on *achiral* ligand scaffolds and spacers, thus ensuring that (enantio)selectivity is provided by second coordination sphere interactions between the catalyst–substrate moiety and the host protein. Having produced, purified and screened several streptavidin (Sav hereafter) mutants, we identified position S112 as a promising mutation site to perform saturation mutagenesis.[23] Combining the twenty Sav S112X with eighteen achiral biotinylated diphosphine ligands afforded ver-



Scheme 1. Second generation artificial metalloenzymes based on the biotin-avidin technology for the reduction of *N*-acetamidodehydroamino acids. The host protein [(strept)-avidin] displays high affinity for the anchor (biotin); introduction of an enantiopure α-amino acid spacer, combined with a flexible diphosphine ligand allows us to chemically optimize both the activity and the selectivity. Site-directed mutagenesis further enables a genetic optimization of the host protein to afford enantioselective artificial hydrogenases.

satile artificial hydrogenases displaying moderate to good enantioselectivities for both enantiomers of *N*-protected amino acids (*N*-acetamidoalanine, *N*-AcAla, and *N*-acetamidophenylalanine, *N*-AcPhe).[34]

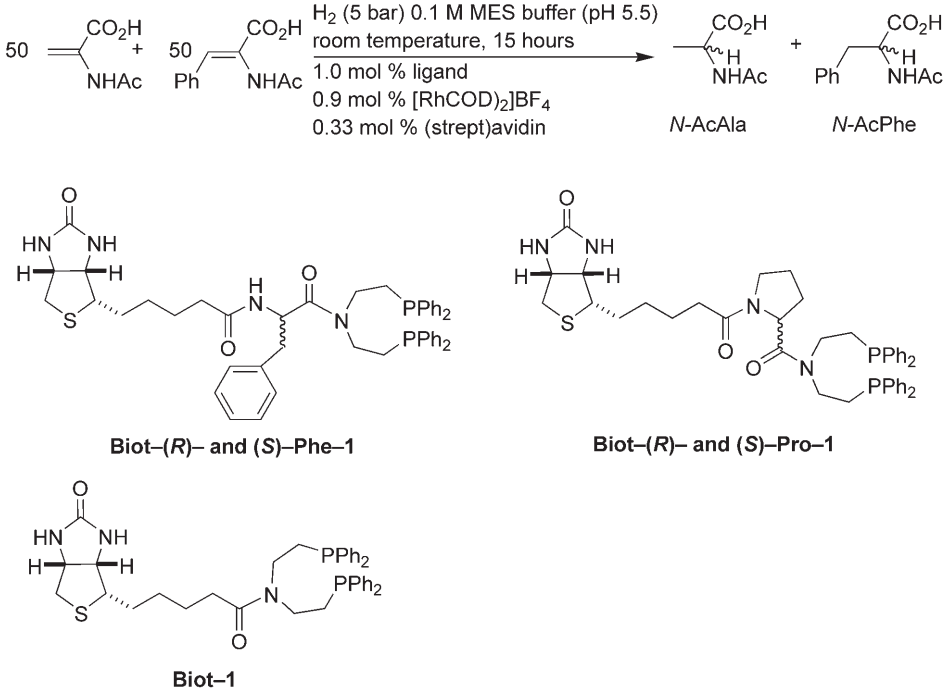
Herein we report on our efforts to produce more active and selective artificial hydrogenases based on the biotin-avidin technology, with an emphasis on the use of enantiopure α-amino acid spacers (Scheme 1). We show that this second generation of hybrid catalysts displays increased stability towards organic solvents, increased selectivity and increased reaction rates. In addition, we show that, by exploiting the tetrameric nature of (strept)avidin [hereafter, (strept)-avidin refers to either avidin or streptavidin], such artificial metalloenzymes can be immobilized using commercially available Biotin-Sepharose with little erosion of their performance. A preliminary account of this work has appeared recently.[26]

Results and Discussion

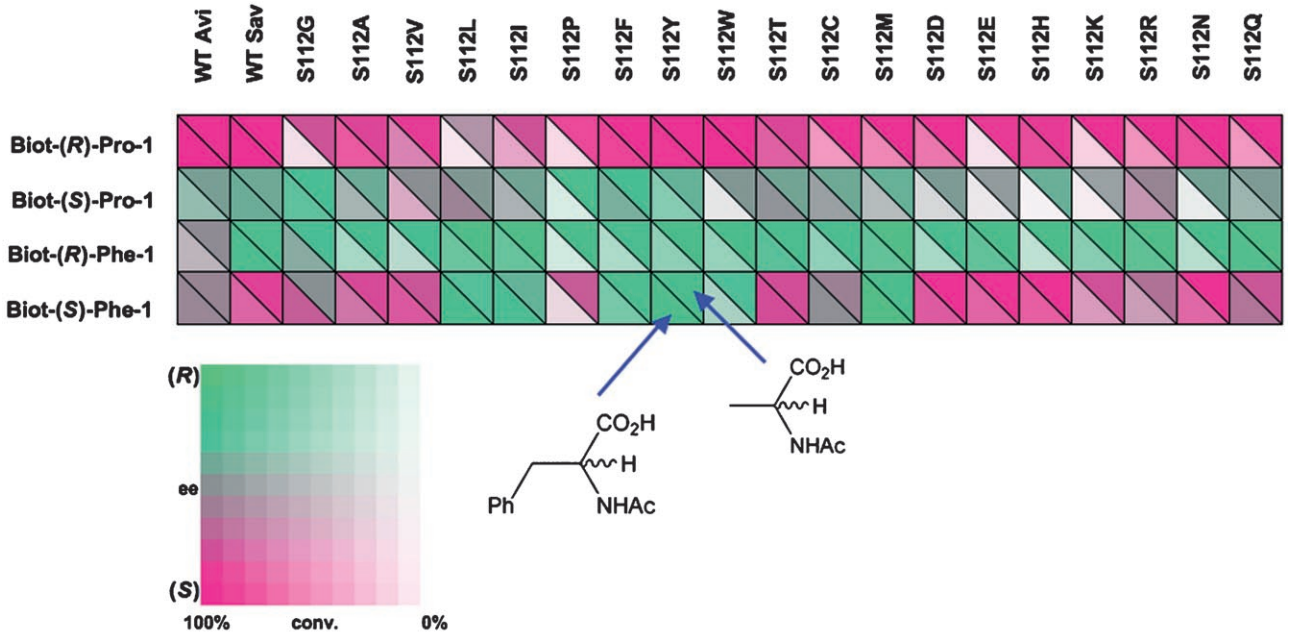
Chemogenetic Diversity

In previous studies, we showed that the ligand scaffold plays a critical role on the performance of the resulting artificial hydrogenases [Rh(COD)(**Biot-spacer-diphosphine**)]$^+$ ⊂ (strept)avidin. For this study, we used exclusively the most versatile ligand scaffold **1** and focused on the influence of the amino acid spacer (Scheme 2).

In order to exploit more efficiently the interactions between the spacer and the (strept)avidin host protein, phenylalanine and proline spacers were selected.



Scheme 2. Biotinylated ligands used in this study and standard operating conditions for the hydrogenation of a cocktail mixture containing α-acetamidoacrylic acid and α-acetamidocinnamic acid (50 equivalents of each with respect to the biotinylated ligand).



Scheme 3. Fingerprint display of the results of the chemogenetic optimization for the reduction of α-acetamidoacrylic acid (top triangle) and α-acetamidocinnamic acid (bottom triangle).

We speculated that the phenylalanine spacer might display stabilizing π–π interactions upon interaction with the hydrophobic (strept)avidin binding pockets, which possess four or five aromatic residues for streptavidin and avidin, respectively. We reasoned that a proline moiety would restrict the degrees of freedom of the biotinylated ligand, thus contributing to localize the catalytic moiety within a well defined position in (strept)avidin.

Both enantiomers of the *Boc*-protected amino acid spacers were coupled to the aminodiphosphine moiety **1**. Following deprotection, (+)-biotin was introduced to afford **Biot-(*R*)-Pro-1**, **Biot-(*S*)-Pro-1**, **Biot-(*R*)-Phe-1**, **Biot-(*S*)-Phe-1**. This N→C coupling scheme avoids epimerization – *via* formation of an intermediate oxazolone – upon the second peptide coupling step. Full experimental details for the ligands synthesis and spectroscopic characterization can be found in the preliminary account of this work.[26]

These ligands were reacted *in situ* with $[Rh(COD)_2]BF_4$ and combined either with WT avidin or with the twenty isoforms of streptavidin obtained by saturation mutagenesis at position S112X. The resulting artificial hydrogenases [Rh(COD)(**Biot-spacer-1**)]$^+$ ⊂ (strept)avidin were screened with a cocktail mixture containing α-acetamidoacrylic acid and α-acetamidocinnamic acid (fifty equivalents of each with respect to the biotinylated ligand), Scheme 2. Control experiments demonstrated that both the selectivity and the conversion obtained with the cocktail mixture are identical to those obtained with a single substrate (i.e., no autoinduction).[35,36]

The results of the chemogenetic optimization are summarized in Scheme 3 using a fingerprint display for each substrate-protein-ligand combination.[25,37] The selectivity is colour-coded [green for (*R*)-selectivity and pink for (*S*)-selectivity] and the intensity of the colour codes for the conversion. Both substrates are displayed as two hypothenuse-sharing triangles to allow a rapid identification of interesting ligand-protein combinations. Selected results are collected in Table 1, and a summary of all catalytic experiments is provided in the Supporting Information.

Inspection of the fingerprint (Scheme 3), reveals several interesting trends.

i) The absolute configuration of the amino acid spacer by-and-large determines the absolute configuration of the reduction product. Both **Biot-(*R*)-Pro-1** and **Biot-(*S*)-Phe-1** favour (*S*)-reduction products, while **Biot-(*S*)-Pro-1** and **Biot-(*R*)-Phe-1** produce preferentially (*R*)-reduction products.

Combining **Biot-(*R*)-Pro-1** with Sav mutants bearing aromatic residues at position S112X affords the most (*S*)-selective artificial hydrogenases (up to 95% *ee* for both substrates, Table 1, entries 3 and 4). It should be emphasized that, in the absence of (strept)avidin, the biotinylated catalysts derived from the enantiopure ligands afford nearly racemic products (<10% *ee* in all cases).

ii) Depending on the amino acid residue at position S112X, the hybrid catalysts derived from **Biot-(*S*)-Phe-1** can afford both (*R*)- and (*S*)-reduction products. For example, [Rh(COD)**Biot-(*S*)-Phe-1**]$^+$ ⊂ Sav S112M and [Rh(COD)**Biot-(*S*)-Phe-1**]$^+$ ⊂ Sav S112H

Table 1. Numerical summary of selected results of the catalytic experiments.

Entry	Ligand	Protein	*ee*[a] of *N*-AcPhe	Conversion of *N*-AcPhe	*ee*[a] of *N*-AcAla	Conversion of *N*-AcAla
1	**Biot-(*R*)-Pro-1**	WT Avi	−89	quant.[b]	−87	quant.[b]
2	**Biot-(*R*)-Pro-1**	S112K	−84	36	−88	quant.[b]
3	**Biot-(*R*)-Pro-1**	S112W	−95	quant.[b]	−95	quant.[b]
4	**Biot-(*R*)-Pro-1**	S112Y	−93	97	−92	quant.[b]
5	**Biot-(*R*)-Phe-1**	S112I	74	80	84	quant.[b]
6	**Biot-(*R*)-Phe-1**	S112L	74	88	83	quant.[b]
7	**Biot-(*R*)-Phe-1**	S112M	75	94	86	quant.[b]
8	**Biot-(*R*)-Phe-1**	S112Q	76	82	81	quant.[b]
9	**Biot-(*R*)-Phe-1**	S112R	82	75	84	quant.[b]
10	**Biot-(*S*)-Phe-1**	S112H	-78	65	-87	quant.[b]
11	**Biot-(*S*)-Phe-1**	S112M	87	quant.[b]	73	quant.[b]

[a] *ee* in %, positive *ee* values in favour of the (*R*)-enantiomer, negative *ee* values in favour of the (*S*)-enantiomer.
[b] Quantitative; all listed experiments were performed at least in duplicate (see Supporting Information); *N*-AcPhe = *N*-acetamidophenylalanine, *N*-AcAa = *N*-acetamidoalanine.

yield quantitatively *N*-AcAla in 73% *ee* (*R*) and 87% *ee* (*S*), respectively (Table 1, entries 10 and 11). For a single mutation, this represents a difference in transition state free energy $\delta\Delta G^{\neq} > 2.6$ kcal mol^{-1} at room temperature. Irrespective of the absolute configuration of the phenylalanine spacer, hybrid catalysts derived from S112F, S112L, S112M, S112W and S112Y streptavidin mutants afford preferentially the same enantiomer. For example, both [Rh(COD)**Biot-(*R*)-Phe-1**]$^+$ ⊂ Sav S112M and [Rh(COD)**Biot-(*S*)-Phe-1**]$^+$ ⊂ Sav S112M yield quantitatively (*R*)-*N*-AcAla in 86% *ee* and 73% *ee*, respectively (Table 1, entries 7 and 11). We speculate that the presence of a bulky residue at position S112 outrules the preference imposed by the enantiopure Phe-spacer. This may be achieved either by inverting the preferred enantioenriched ligand conformation or by forcing the prochiral substrate to present its *si*-face to the biotinylated catalyst.

iii) Both substrates afford comparable enantioselectivities. However, slightly better conversions are generally obtained for the smaller product *N*-AcAla. Significant substrate discrimination is found for catalysts derived from **Biot-(*R*)-Pro-1**. For example, [Rh(COD)**Biot-(*R*)-Pro-1**]$^+$ ⊂ Sav S112K affords quantitatively (*S*)-*N*-AcAla (88% *ee*), while the conversion for the larger (*S*)-*N*-AcPhe remains modest (36% conversion, 84% *ee*, Table 1, entry 2)

iv) Combining **Biot-(*R*)-Pro-1** with WT Avi affords, for the first time, good levels of enantioselection (89% *ee* for *N*-AcPhe quantitative, Table 1 entry 1). This may be of interest for future developments as WT Avi is significantly cheaper that WT Sav.

In summary and in comparison to our previous studies relying on achiral spacers,[22–25,34] the introduction of enantiopure spacers yields higher (*S*)-selective artificial metalloenzymes. In addition, the genetic optimization by saturation mutagenesis at position S112X Sav generates more diversity than previously observed with achiral spacers.

Organic Solvent Tolerance and Catalyst Immobilization

One of the main limitations of enantioselective catalysis performed in aqueous media is the very limited solubility of many substrates. The organic solvent tolerance should thus contribute to broaden the substrate scope of these artificial metalloenzymes.

Having identified both (*R*)- and (*S*)-selective artificial hydrogenases (*ee* > 90%), we proceeded to test these hydrogenases in the presence of large amounts of organic co-solvents. For this purpose, dimethyl sulfoxide (DMSO, miscible with water) and ethyl acetate (EtOAc, non-miscible with water) were selected. The results for the screening in the presence of the most selective catalyst ⊂ protein combinations are summarized in Table 2.

As can be appreciated from these data, the catalyst derived from **Biot-1** does not tolerate large amounts of either DMSO or EtOAc (Table 2, entries 1 and 2).[26] Introduction of an enantiopure spacer confers significant organic-solvent tolerance in the presence of streptavidin and its mutants. Both (*S*)- and *(R)*-selective catalysts [Rh(COD)**Biot-(*R*)-Pro-1**]$^+$ ⊂ Sav S112W and [Rh(COD)**Biot-(*S*)-Phe-1**]$^+$ ⊂ Sav S112M perform well in the presence of either 45% DMSO or EtOAc (maximal *ee* erosion 12%, Table 2, entries 12 and 13, 15 and 16). In contrast to streptavidin isoforms as host proteins, avidin-based artificial metalloenzymes perform poorly in the presence 45% DMSO (Table 2, compare entries 4/5 and 8/9). In the presence of either 27% DMSO or EtOAc, the erosion observed with [Rh(COD)**Biot-(*R*)-Pro-1**]$^+$ ⊂ WT-Avi is minimal (Table 2, entries 8 and 10).

Table 2. Numerical summary of selected results of the catalytic experiments performed in the presence of organic solvents or with immobilized artificial metalloenzymes.

Entry	Ligand	Protein	Organic solvent	*ee*[a] of *N*-AcPhe	Conversion of *N*-AcPhe	*ee*[a] of *N*-AcAla	Conversion of *N*-AcAla
1[b]	**Biot-1**	WT Sav	45% DMSO	24	9	16	71
2[b]	**Biot-1**	WT Sav	EtOAc	31	5	30	56
3	**Biot-1**	WT Sav	Immob.[d]	93	78	83	quant.[c]
4	**Biot-(*R*)-Pro-1**	WT Sav	27% DMSO	−91	100	−87	quant.[c]
5[b]	**Biot-(*R*)-Pro-1**	WT Sav	45% DMSO	−86	94	−87	quant.[c]
6[b]	**Biot-(*R*)-Pro-1**	WT Sav	EtOAc	−87	85	−83	90
7	**Biot-(*R*)-Pro-1**	WT Sav	Immob.[d]	−89	36	−76	76
8	**Biot-(*R*)-Pro-1**	WT Avi	27% DMSO	−82	94	−86	quant.[c]
9	**Biot-(*R*)-Pro-1**	WT Avi	45% DMSO	−15	34	−37	quant.[c]
10	**Biot-(*R*)-Pro-1**	WT Avi	EtOAc	−89	77	−87	quant.[c]
11	**Biot-(*R*)-Pro-1**	WT Avi	Immob.[d]	−80	32	−76	quant.[c]
12	**Biot-(*R*)-Pro-1**	S112W	45% DMSO	−88	56	−85	quant.[c]
13	**Biot-(*R*)-Pro-1**	S112W	EtOAc	−94	86	−94	quant.[c]
14	**Biot-(*R*)-Pro-1**	S112W	Immob.[d]	−94	89	−92	quant.[c]
15	**Biot-(*S*)-Phe-1**	S112M	45% DMSO	75	68	69	quant.[c]
16	**Biot-(*S*)-Phe-1**	S112M	EtOAc	81	78	64	quant.[c]
17	**Biot-(*S*)-Phe-1**	S112M	Immob.[d]	81	50	33	quant.[c]

[a] *ee* in %, positive *ee* values in favour of the (*R*)-enantiomer, negative *ee* values in favour of the (*S*)-enantiomer.
[b] Data from ref.[26]
[c] Quantitative.
[d] Immobilized using Biotin-Sepharose; all listed experiments were performed at least in duplicate (See Supporting Information); *N*-AcPhe = *N*-acetamidophenylalanine, *N*-AcAla = *N*-acetamidoalanine.

In the past, we have shown that an artificial metalloenzyme's selectivity is little affected upon varying the ratio of [Rh(COD)**Biot-1**]$^+$ *versus* WT Sav between one and four, suggesting that the occupancy of one binding site does not significantly affect neighbouring sites.[24] As streptavidin is a homo-tetrameric protein, we thus speculated that it may be possible to exploit one binding site to immobilize the artificial metalloenzyme, leaving three sites for the incorporation of a biotinylated catalyst.

For this purpose, one equivalent of commercially available Biotin-Sepharose was added to tetrameric (strept)avidin prior to the addition of an excess biotinylated catalyst precursor. After decanting the immobilized catalyst precursors, thus removing the excess biotinylated rhodium present in solution, these were tested using the standard reaction conditions. Selected results are collected in Table 2. As can be appreciated, the immobilized artificial metalloenzymes are most often nearly as selective as their homogeneous counterparts. However, the activity decreases, especially for the larger α-acetamidocinnamic acid substrate. The most versatile immobilized hybrid catalyst is [Rh(COD)**Biot-(*R*)-Pro-1**]$^+$ ⊂ Sav S112W which is as active and as selective as its homogeneous counterpart (compare Table 1 entry 3 and Table 2 entry 14).

In the context of artificial metalloenzymes, recovering the precious protein *via* a simple filtration step offers a straightforward work-up procedure. In addition, a second catalytic run performed with this recycled immobilized artificial metalloenzyme afforded similar conversions and enantioselectivity compared to the first catalytic run. For [Rh(COD)**Biot-(*R*)-Pro-1**]$^+$ ⊂ Sav S112W) with α-acetamidocinnamic acid, *ee* 94% (*S*), conversion 81%, compare with Table 2 entry 14. The third catalytic run however afforded a significantly lower yield and enantioselectivity (*ee* <30%, conversion <25%).

Michaelis–Menten Kinetics

Having identified efficient artificial metalloenzymes, we proceeded to determine the Michaelis–Menten parameters for four catalytic systems: [Rh(COD)**Biot-1**]$^+$, [Rh(COD)**Biot-1**]$^+$ ⊂ WT Sav, [Rh(COD)**Biot-(*R*)-Pro-1**]$^+$ ⊂ WT Sav and [Rh(COD)**Biot-(*R*)-Pro-1**]$^+$ ⊂ WT-Avi. In order to test the influence of the organic co-solvent, catalysis with the latter two hybrid catalysts was performed in 30% DMSO, thus ensuring good levels of selectivity both for streptavidin and for avidin based systems (compare entries 4/5 and entries 8/9, respectively, in Table 2). For convenience, the reactions were carried out at 2 bar hydrogen pressure and room temperature. For solubility reasons, only the more soluble substrate *N*-acetamidoacrylic

acid was used. The results are displayed in Figure 1 and summarized in Table 3.

These data reveal several noteworthy features:

i) All catalytic systems display Michaelis–Menten behaviour.

ii) Compared to the protein-free catalyst (Table 3, entry 1) all artificial metalloenzymes display higher affinity for the substrate (i.e., smaller K_M, Table 3, entries 2–4).

iii) Compared to the protein-free catalyst (Table 3, entry 1), all artificial metalloenzymes display higher pseudo-first-order reaction rates (Table 3, entries 2–4).

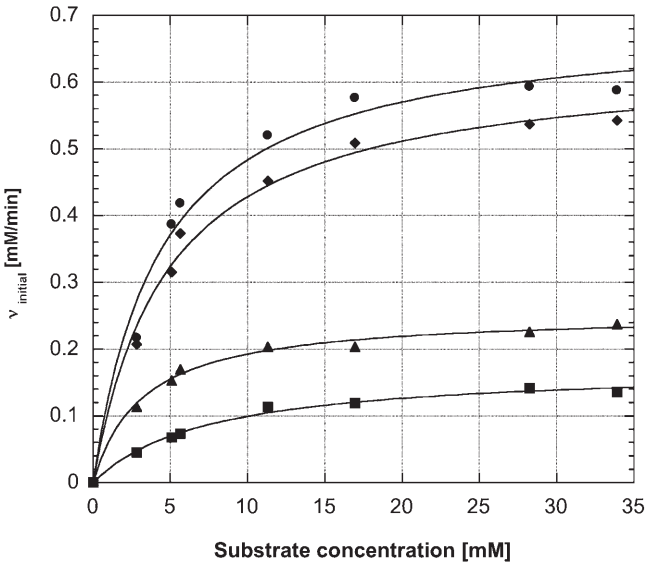


Figure 1. Saturation kinetics plots for the reduction of *N*-acetamidoacrylic acid with [Rh(COD)**Biot-1**]$^+$ (9% DMSO, measured: squares), [Rh(COD)**Biot-1**]$^+$ ⊂ WT Sav (9% DMSO, measured: triangles), [Rh(COD)**Biot-(*R*)-Pro-1**]$^+$ ⊂ WT Sav (30% DMSO, measured: circles) and [Rh(COD)**Biot-(*R*)-Pro-1**]$^+$ ⊂ WT Avi (30% DMSO, measured: diamonds). All the fitted graphics are shown in solid lines.

Table 3. Michaelis–Menten parameters for the reduction of *N*-acetamidoacrylic acid with selected artificial hydrogenases at 2 bars H_2 and room temperature.

Entry	Ligand	Protein	V_{max} [mM min^{-1}]	k_{cat} (min^{-1})	K_M [mM]
1	**Biot-1**	-	0.173	3.06	7.38
2	**Biot-1**	WT Sav	0.254	4.49	3.18
3	**Biot-(*R*)-Pro-1**	WT Sav	0.695	12.30	4.36
4	**Biot-(*R*)-Pro-1**	WT Avi	0.634	11.23	4.80

iv) Both avidin and streptavidin host proteins display similar kinetic behaviour (Table 3, entries 3 and 4).

v) The presence of large quantities of DMSO contributes to significantly increase the reaction rates (Table 3, entries 3 and 4).

It thus appears that both the affinity of the catalyst for the substrate and the turnover rates are increased with respect to the protein-free reference system [Rh(COD)**Biot-1**]$^+$. A possible reason may be the presence of a hydrophobic cavity provided by the protein, which contributes to increase the local concentration of both the substrate and of the dihydrogen close to the catalytic moiety. Accumulation within the host protein of both substrate and dihydrogen are reflected in lower K_M and higher k_{cat}, provided that the H_2 oxidative addition is the rate-determining step.[38]

Conclusions

This study, based on the incorporation of enantiopure amino acid spacers between the biotin anchor and the diphosphine moiety, considerably extends the potential of artificial hydrogenases based on the biotin avidin technology. The most noteworthy features revealed herein include:

i) Both avidin and streptavidin can be used as host proteins to afford selective hybrid catalysts. For the first time, egg-white avidin – which is considerably cheaper than streptavidin – affords reduction products with *ee* >86% in favour of the (*S*)-enantiomer using [Rh(COD)**Biot-(*R*)-Pro-1**]$^+$ ⊂ WT Avi. The chemogenetic optimization with streptavidin mutants, affords hybrid catalysts for both *(R)*- and (*S*)-reduction products with *ee*s >90%: for example, [Rh(COD)**Biot-1**]$^+$ ⊂ WT Sav and [Rh(COD)**Biot-(*R*)-Pro-1**]$^+$ ⊂ S112W Sav.

ii) In general, the nature of the spacer by-and-large determines which enantiomer is preferentially produced. However, hybrid catalysts derived from **Biot-(*S*)-Phe-1**, afford both (*R*)- and (*S*)-reduction products with *ee* >75%, depending on the nature of the point mutation at position S112.

iii) Increased organic solvent tolerance: the use of large amounts of DMSO (up to 45%) may allow one to solubilize hydrophobic substrates, while EtOAc allows a straightforward work-up to recover the substrate.

iii) Immobilization using Biotin-Sepharose with little erosion of the selectivity: again here, the work-up is considerably facilitated.

iv) Michaelis–Menten behaviour with increased rate compared to the biotinylated catalyst devoid of (strept)avidin.

Current efforts in this area are centred on the reduction of challenging dehydroamino acid substrates (with limited aqueous solubility) as well as increasing

the turnover numbers and turnover frequency of artificial hydrogenases based on the biotin-avidin technology.

Experimental Section

Ligand Synthesis and Characterization

All ligands used herein were reported (synthesis and full characterization) in the supporting information of the preliminary communication.[26]

Production and Purification of Streptavidin

The expression of mutant proteins Sav S112X has been described elsewhere.[34] WT Avi was provided by Belovo.

Hydrogenation Reactions

General Aspects: All aqueous and organic solvents were flushed with nitrogen during at least three hours. All manipulations were performed in a glove-box under a nitrogen atmosphere. The metal source $[Rh(COD)_2]BF_4$ was dissolved in DMSO (Acros) (4 mg, 16 mL DMSO), 1.56 μmol of this solution were added to the appropriate aliquoted ligand (2.02 μmol, 1.3 equivs. with respect to the metal source). This solution was stirred for ca. 10 min.

Hydrogenation in Monophasic Media (9%, 27%, 45% DMSO): A Pyrex tube (volume *ca.* 3 mL) was placed in an autoclave and charged with an *N*-acetamidoacrylic acid solution in MES buffer 0.38 M (130 μL of a 23.85 mM solution, 3.10 μmol), and an *N*-acetamidocinnamic acid solution in MES buffer 0.38 M (130 μL of a 23.85 mM solution, 3.10 μmol). The volume was adjusted with water (or with DMSO, depending on the ratio of DMSO to be used) to 900 μL. The protein solution in water was then added (100 μL of a 0.207 mM solution, 0.0207 μmol of the tetramer). The precatalyst solution in DMSO (100 μL, 0.062 μmol) was added last. The resulting mixture was vortexed, removed from the glove-box and hydrogenated at 5 bars H_2 for 15 h.

Hydrogenation in Biphasic Media (EtOAc): A Pyrex tube (volume *ca.* 3 mL) was placed in an autoclave and charged with an *N*-acetamidoacrylic acid solution in EtOAc (550 μL of a 5.66 mM solution, 3.10 μmol), and an *N*-acetamidocinnamic acid solution in EtOAc (550 μL of a 5.66 mM solution, 3.10 μmol). The buffer solution (580 μL of a 0.38 M solution) was added and the volume of the aqueous phase adjusted with water to 900 μL. The protein solution in water was then added (100 μL of a 0.207 mM solution, 0.0207 μmol of the tetramer). The precatalyst solution in DMSO (100 μL, 0.062 μmol) was added last. The resulting mixture was vortexed and hydrogenated at 5 bars H_2 for 15 h using orbital shaking (*ca.* 200 rpm).

Note: In the case of hydrogenation without protein, the volume of the protein solution was replaced by H_2O.

All experiments were performed using MES buffer only. This change does not influence significantly the activity and enantioselectivity of experiments using avidin as the host protein.

Hydrogenation Procedure for Immobilized Artificial Metalloenzymes: The commercial suspension of D-biotin-sepharose CL-4B {[105 μL, which corresponds to 0.031 μmol biotin, capable of immobilizing 0.031 μmol tetrameric (strept)avidin], [Affiland, Belgium, suspension in TBS buffer pH 7.4, NaN_3 0.02% (w/v)]} was decanted and washed with water to afford wet beads, which were resuspended in water (105 μL total volume). The protein solution (0.062 μmol, 2 equivs. *vs.* biotin sepharose, 300 μL) was added. After *ca.* 10 min incubation, the immobilized protein (containing 0.031 μmol of protein, corresponding to 0.093 μmol, free active sites) was washed at least three times with H_2O to remove the unbound protein and flushed with nitrogen for three hours. The precatalyst solution in DMSO (100 μL, 0.062 μmol, 0.66 equivs. with respect to the free biotin-binding sites) was added last and was incubated with the immobilized protein for 10 min. The immobilized catalyst was subjected to several cycles of washing and decanting. The catalytic runs were performed according to the standard orbital shaking procedure.

Hydrogenation Procedure for the Determination of Michaelis–Menten Parameters: A 15-mL glass vessel was charged with a quantity (depending on the number of equivalent of substrates used) α-acetamidoacrylic acid in MES buffer (0.1 M, pH 5.5). The volume was adjusted with water to 9 mL followed by addition of (strept)avidin in H_2O (1 mL, 0.207 mM solution, 0.207 μmol of tetramer). The catalyst precursor $[Rh(COD)(spacer)]^+$ in DMSO (1 mL, 0.62 μmol) was added last. The resulting mixture was stirred for *ca.* 2 mins to ensure a homogeneous medium. The reactor was closed and was removed from the glove box and pressurized to 2 bars H_2 and stirred mechanically at room temperature. Aliquots (*ca.* 0.5 mL) were sampled at given times.

The initial conversions were collected for each concentration of substrate. The initial rate of the reaction (**v**) can be calculated by:

$$v = ([\text{substrate}]_0 \cdot \text{conversion})/t.$$

This value gives the observed initial rate. The calculated initial rate can be obtained by employing the following equation:

$$v = \frac{V_{max}[S]}{K_M + [S]} \qquad V_{max} = k_{cat}[E]_0$$

with:

v = initial rate of reaction

V_{max} = maximum rate of reaction (kinetic saturation)

[S] = concentration of substrate

$[E]_0$ = initial concentration of enzyme (catalyst)

K_M = Michaelis–Menten (dissociation of substrate-catalyst complex) constant

k_{cat} = rate constant (turnover number) of reaction.

The initial guessed values of both unknowns (K_M and V_{max}) were introduced in the calculation. Then, a least squares-minimization was performed to obtain the calculated initial rate v of the reaction and the Michaelis–Menten parameters.

Supporting Information

A summary of all catalytic runs is provided free of charge via the internet or from the author.

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