Designed evolution of artificial metalloenzymes: protein catalysts made to order

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Artificial metalloenzymes based on biotin–streptavidin technology, a "fusion" of chemistry and biology, illustrate how asymmetric catalysts can be improved and evolved using chemogenetic approaches.

Introduction

The year 2007 marks the 100th anniversary of Eduard Buchner winning the Nobel Prize in Chemistry (1907) for describing that dead extracts of yeast could catalyse the fermentation of sugars into alcohol. Buchner's seminal discovery, published just 10 years earlier,[1] hinted that even the complex reactions of life had an underlying chemical basis. The debate of chemical *versus* enzyme catalysis raged for years within the scientific community, as demonstrated in a report by the National Research Council of 1928:[2]

"*At times it has been held by some that the two divisions of chemical action have little or nothing in common beyond certain superficial resemblances*; *others have maintained the opposite view*, *and believed that fermentation and inorganic catalysis were varying instances of the same mode of operation of chemical activity*."

However, it was soon accepted that chemical catalysis has much in common with nature's own catalysts: enzymes. It is beyond doubt that Buchner greatly contributed to the modern interdisciplinary fusion between chemistry and biology. Further, Buchner's discovery may be considered in retrospect to mark the birth of "biomolecular chemistry". Indeed, by the 1950s, the synergism of homogeneous catalysis and enzyme chemistry was demonstrated in the first use of "model substances having enzyme-like activity" to investigate the mode of action of natural enzymes.[3]

Our increased understanding of chemical and enzymatic catalysis over the last 100 years, especially since the advent of genetic engineering and recombinant technology, has led the scientific community to attempt the development of new enzymes and catalysts with modified activities, specificities and activities.

Many types of catalysts have been described and, although it is beyond the scope of this article to present them comprehensively, they can be categorized as heterogeneous, organometallic, organic or enzymatic.[4–7] This article is chiefly concerned with one area

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Terminology

Artificial metalloenzymes, as used here, are catalysts resulting from the introduction of a metal ion-complex with catalytic activity into macromolecular hosts, such as a protein or DNA, which control the second coordination sphere and thus the selectivity of the reaction.

Protein scaffold is the 3-D structure formed by motifs and domains that serves as a framework for evolution of protein activities, including the evolution of catalytic activity through the modification of key amino acids. The (strept)avidin protein scaffold has been extensively used as a host for artificial metalloenzymes: the genetic modification of the (strept)avidin scaffold forms the basis for the designed evolution of these artificial metalloenzymes.

Protein fitness is a measure of protein function as defined by particular properties (normally beneficial properties that are sought), such as catalytic activity, enantioselectivity, thermostability or tolerance to organic solvents. Fitness is often measured by comparison to the wild-type protein: *e.g.* a variant may have a 300% increase in catalytic activity compared with the wild-type.

Protein sequence space is defined as all the possible protein neighbours that can be obtained by a series of single point mutations. For example, an *n* residue long protein would be an *n* dimensional object with 20 possible values (the 20 naturally occurring amino acids) in each dimension.

Protein fitness landscape is, conceptually, the protein-fitness of each protein in sequence space. It is noteworthy that the sequence space of proteins is immense and that its associated fitness landscape is, in practice, impossible to explore exhaustively.

Functionally enriched protein-space is the region of protein space where the proportion of proteins with the desired fitness is greatly increased compared with surrounding regions.

Red biotechnology is biotechnology applied to medicine, such as the use of genomic manipulation for the treatment and cure of inherited diseases.

White biotechnology is biotechnology applied to industrial processes, such as the genetic engineering of an enzyme or an organism to produce a useful chemical. White biotechnology tends to consume less in resources that traditional processes when used to produce industrial goods.

in which the fields of organometallic catalysis and enzyme chemistry overlap: hybrid catalysts. Hybrid catalysts result from the complementation of biomolecules (*e.g.* proteins or DNA) and organometallic catalysts to form increasingly sophisticated artificial enzymes.[8] In specific, this particular "fusion" of chemistry and biology will be illustrated by focusing on artificial metalloenzymes in the field of asymmetric catalysis.

Enantioselectivity of homogeneous catalysts

Some non-biological metals such as palladium, rhodium and ruthenium have been used for many years in synthetic chemistry as very efficient homogeneous catalysts. A pioneering example was the discovery by Wilkinson *et al.* that chlorotris-(triphenylphosphine)rhodium [RhC1(PPh_3)$_3$], was a soluble hydrogenation catalyst for unhindered olefins.[9] Shortly thereafter, William S. Knowles extended the use of Wilkinson's transition catalysts to asymmetric hydrogenation, showing that transition metals could be placed within a chiral environment (provided by enantiopure ligands) to carry out asymmetric catalysis (Fig. 1C).[10] Knowles rationalised the phenomenon of asymmetric catalysis by using Pauling's postulate[11] that the chiral catalyst stabilised one diastereomeric transition state over the other, leading to preferred formation of one enantioenriched product over the other.

Many other examples of asymmetric metal-catalysis have been developed since,[4] including those of Noyori *et al.*[12,13] and Sharpless.[14] However, is it possible to rationally design enantioselective catalysts? Knowles himself acknowledged the difficulty of this task:[15]

"*Since achieving 95% ee only involves energy differences of about 2 kcal, which is no more than the barrier encountered in a simple rotation of ethane, it is unlikely that before the fact one can predict what kind of ligand structures will be effective.*"

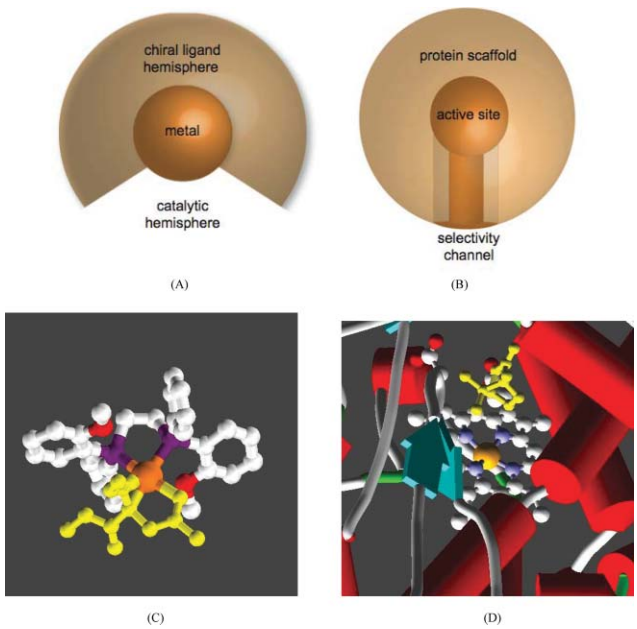

Fig. 1 Schematic representation of an enantioselective homogeneous catalyst (A) and of an enzyme (B); structure of Knowles's $\{Rh(DIPAMP)\}^+$ bound to its substrate (yellow), emphasizing the limited space (hemisphere) occupied by the enantiopure ligand (CSD ref. code: VERYAS). Note the orientation of the phenyl groups bound to phosphorus: the *o*-anisyl presents its face while the phenyl group presents its edge (C); structure of cytochrome P450 cam with its substrate (camphor, yellow), emphasizing the "lock and key" complementarity between the enzyme and its substrate (pdb ref. code: 2CPP) (D).

Can one routinely design catalysts that give *predictably* enantioenriched products? One should hesitate to provide a "yes" as an answer in the foreseeable future, because subtle experimental parameters (solvent, counterion, added salts, and so forth), which influence weak contacts between a catalyst and its "nonbonded" environment (the second coordination sphere) often have a significant and unpredictable influence on the enantioselectivity of a reaction.[16] Nevertheless, those asymmetric catalysts that are most successful must exert considerable control over the second coordination sphere of a reaction, presumably by defining the local factors that determine the outcome of catalysis whilst "shielding" the active site from variable experimental parameters. We speculate that, whereas the catalyst may exert considerable control over one of the hemispheres around the catalytically active metal (*i.e.* chiral ligand hemisphere), the limited size and "reach" of many enantiopure ligands restricts their influence over the more "distant" hemisphere where the enantiodiscriminating event occurs (Fig. 1A). Therefore, the most successful catalysts may well be those that control the largest part of the catalytic hemisphere. However, even with a tight control of the entire second coordination sphere, the extent and direction of enantioenrichment of a catalytic reaction will remain hard to predict, for which a combinatorial and screening approach is warranted for the identification of new asymmetric catalysts.[17–19]

Recapitulating, two conclusions arise: (1) the size and "reach" of catalysts may be a limiting factor for maximizing control of the second coordination sphere and (2) due to the unpredictability of enantiodiscrimination, a screening approach is likely to continue to be necessary for the identification of new asymmetric catalysts.

It is interesting to note that one of the first enantioselective hydrogenation catalysts was derived from impregnating silk with palladium dichloride with a subsequent reduction step. The resulting hybrid (heterogeneous) catalyst, formed by embedding the palladium within a large, insoluble assembly of fibrous proteins, gave rise to appreciable ee's (up to 25% for phenylalanine);[20] this may well be the first example of an artificial metalloenzyme.

Enantioselectivity of enzymes

It is estimated that around one third of all enzymes are metalloproteins and that some of the most difficult biological reactions are mediated by these.[21] In addition to supplying a chiral first coordination sphere, the protein scaffold is exquisitely suited to provide a well defined second coordination sphere. Evolution appears to have tailored the 2nd coordination sphere of enzymes to maximise the efficiency and the selectivity of the catalyzed reactions. For this purpose, the catalytic site of many enzymes can be accessed only *via* a narrow channel (which we coin a "selectivity channel," reminiscent of the potassium channels),[22] which ensures selectivity (Fig. 1B and 1D). This latter feature is particularly difficult to master in homogeneous catalysis and a source of much frustration in catalyst design.

State-of-the-art protein chemistry has reached the point where we can "evolve" enzymes at a whim by directed evolution, rationally modify the activity of enzymes and even attempt to design metal-containing enzymes *de novo.*[23–26] Notwithstanding recent progress in explaining the mechanism and selectivity of enzymes,[27–29] will we be able routinely to design or evolve enzymes that satisfy all of our requirements? The challenge lies in the immenseness of biomolecular space, in the intrinsic constraints of biological molecules (such as a limited array of reactive groups) and in the added difficulty of controlling several characteristics simultaneously (for example, finding an enzyme with high thermal stability and high catalytic activity for the generation of an enantioenriched product). These challenges are likely to slow the success of enzyme design and evolution.

To circumvent some of the current problems in asymmetric catalysis, a promising complementary approach to the barrage of new technologies is the use of hybrid catalysts, which make use of the unmatched activity of non-biological catalysts, together with the potential for chiral enantioselection of a biomolecular scaffold.

Enantioselectivity of artificial metalloenzymes

In the case of asymmetric synthesis using artificial hybrid metalloenzymes, the homogeneous catalyst is a metal complex, whereas the chiral environment is provided by a macromolecular host or "scaffold" (typically a protein[8,30] or nucleic acid[31–33]). The catalyst can be bound covalently to the protein scaffold,[34–38] as well as non-covalently (Fig. 2). A well-known special case of non-covalent binding is the use of antibodies against the catalyst as the biological host;[39–41] however, recently antibodies have also been linked covalently to form "antibodies with infinite affinity", providing the potential to make improved antibody-based artificial metalloenzymes.[42] An excellent review of alternative supramolecular complexes for transition metal-catalysis appeared in this journal recently.[43]

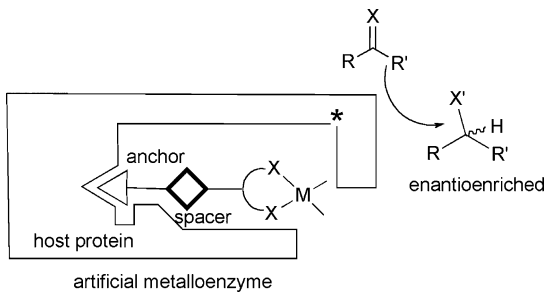

Fig. 2 General scheme of artificial metalloenzymes for enantioselective catalysis based on the incorporation of a catalytically active metal fragment within a host protein. Chemical optimisation can be achieved either by varying the spacer (square) or the metal chelate moiety (MX_2). Saturation mutagenesis at a position close to the metal moiety (*) can be used for genetic optimisation.

What are the main advantages of using artificial metalloenzymes?

New catalytic properties

Non-biological metals, such as those used in many homogeneous metallocatalysts, display catalytic properties that are seldom present in enzymes or that could not be easily accomplished, if at all, using conventional biochemistry. Enantioselective hybrid catalysts have already been designed for a wide range of reactions, including ester hydrolysis,[44] hydroxylation,[45] epoxidation,[46,47] sulfoxidation,[48–52] hydrogenation,[30,41,53–59] transfer hydrogenation,[60,61] and Diels–Alder reactions.[31,32,62]

Protein design

It is much harder to create a stable enzyme *de novo* than to evolve a catalytic function from an existing scaffold. Artificial metalloenzymes take advantage of existing protein scaffolds with attractive properties, to which a new catalytic activity is added extraneously.

Enantiodiscrimination

As discussed above, in homogeneous catalysis, the chiral ligand occupies one hemisphere and the enantiodiscriminating event occurs on the opposite hemisphere. In contrast, in most metalloenzymes, the metal is embedded within a chiral cavity (compare Fig. 1A and 1C with Fig. 1B and 1D). The macromolecular nature of artificial metalloenzymes is expected to lead to a more efficient chiral discrimination than a homogeneous catalyst alone, much in the spirit of a glove (the protein) being able to distinguish a hand (the substrate).

Functional discrimination

In asymmetric catalysis, the artificial enzyme activity is largely determined by the metal complex, whereas the enantioselectivity is largely determined by the protein environment. The unique separation of catalyst and host-protein allows distinct control of functional characteristics with little effect on the other, such as selection of a thermostable scaffold and an active catalyst, independently. In contrast, in enzyme design and evolution, characteristics are often linked and, for example, there are cases where evolution of thermostability may lead to lowered activity.[63]

It is noteworthy that the protein scaffold of a hybrid catalyst may lead to protein-induced increases in the rate of catalysis, perhaps by providing a "hydrophobic cavity"; an asymmetric conformation induced by the second coordination-sphere interactions of the ligand–metal complex may in itself lead to considerable enantioselectivity. Finally, there may exist interactions of functional groups from the protein framework with the "cofactor" for covalent catalysis.[64]

The origins of artificial enzymes based on biotin-(strept)avidin technology

In the 1970s, Whitesides and Wilson[53] pioneered the concept of introducing a homogeneous catalyst within a protein environment to guide enantioselectivity (Fig. 2). Whereas Knowles's innovative approach consisted of finding a useful and well-known catalytic activity and to provide this catalyst with a chiral environment to direct enantioselectivity, Whitesides' insight consisted in the biomimetic approach of embedding a homogeneous catalyst within a protein environment, to form a hybrid catalyst. Using this system for hydrogenation, modest but definite enantiomeric excesses of products were achieved (44% ee).

By following the pioneering work of Whitesides,[53] we endeavour to use the modern tools of genetic engineering to control the second coordination sphere of the homogeneous catalyst.[57] Whereas genetic engineering has been enormously successful in elucidating and modifying enzyme mechanisms, including for the directed evolution of the enantioselectivity of enzymes, one of us was the first to show that an artificial metalloenzyme could be modified genetically[54] to improve its "fitness" (in this case, its enantioselectivity), thus paving the path for further directed evolution of artificial metalloenzymes.[59] The general strategy that we employed initially was a "chemogenetic approach",[64] whereby both the various components of the metal complex (*e.g.* the metal, the first coordination sphere and the "spacer" linking it to the protein) as well as the protein "scaffold" were subjected to optimization (Fig. 3).

An evolving story: the concept of designed evolution of artificial enzymes

The chemogenetic approach to artificial enzymes has the potential for providing hybrid protein catalysts "made to order". Our preferred methodology is a combination of rational design and combinatorial screening[66] leading to the "evolution" of the enzyme, for which we borrow the term *designed evolution*.[67] Designed evolution incorporates the need for rational decisions on choices

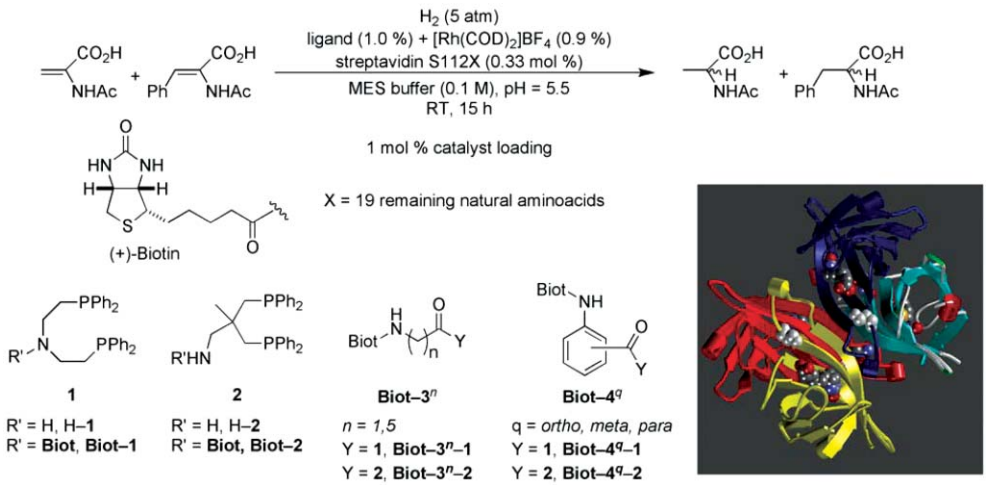

Fig. 3 Operating conditions used for the chemogenetic optimization of artificial hydrogenases for the reduction of α-acetamidoacrylic acid and α-acetamidocinnamic acid, including two of the ligands used for the catalysis results shown in Fig. 5. Adapted from Klein *et al.* (2004).[65] Right insert: structure of tetrameric streptavidin with biotin bound (space-filling model); residue S112 is highlighted in white.

of scaffolds and elements to combine, followed by several rounds of screening to perfect those elements that cannot be predicted *a priori*.

One of the first examples reported in the literature for a design of the power of combining rational and evolutionary design was the failed attempt to develop a new catalytic activity using an α–β-barrel scaffold, converting an indole-3-glycerol synthase into a phosphoribosylanthranilate isomerase.[68] Recently, Park *et al.* have successfully used a similar approach, with emphasis on a "modular" design, for the introduction of a β-lactamase activity into the αβ–βα-metallohydrolase scaffold of glyoxalase.[69]

When designed evolution is applied to hybrid metalloenzymes, the first decision in the scheme is an appropriate choice of homogeneous catalyst (Fig. 3 and 4). For example, it is well established that Ru, Rh and Ir complexes can catalyse the hydrogenation of alkenes. The general reaction conditions (*e.g.* temperature, buffer, pH) may be known or can be defined using the homogeneous catalyst alone. The second decision is the choice of second coordination sphere (protein scaffold) that is compatible with reaction conditions. It is noteworthy that the reaction conditions, such as a requirement for high temperatures or extreme pH, may impose evolutionary restrictions on the protein scaffold and that for extensive screening it is preferable to start with an appropriately robust scaffold.[70,71] Due to the number of possible combinations of protein hosts, it is also convenient to limit the variations to a few designed choices: *e.g.* avidin and streptavidin. Further, in the absence of selection or high-throughput screening methods, then amino acid residues closest to the active site and within the range of the "second coordination sphere" (*e.g.* 10 Å) can be mutagenised.[72,73] Finally, chemogenetic screening is used to identify hybrid variants with improved "fitness", such as high ee and selectivity for substrate at a chosen turnover number.

It is envisaged that as we learn more about the reaction mechanisms of these particular hybrid catalysts, particularly as concerns the second coordination sphere, we will be able to make wiser choices for "designed evolution", optimally leaving the really "fine-tuning" to genetic, evolutionary approaches (Fig. 4).

An example of designed evolution: artificial hydrogenases based on biotin-(strept)avidin

Our experience in artificial metalloenzymes has shown that the chemogenetic "fine-tuning" of the second coordination sphere can have dramatic effects on selectivity, even leading to an inversion of enantioselectivity (Fig. 5).

In the first published example from our lab (Fig. 5), the hydrogenation of acetamidoacrylic acid using diphosphine rhodium complexes [Rh(**Biot-1**)COD]$^+$, yielded 94% ee in favour of the (*R*)-product when using wild-type streptavidin, whereas this enantioselectivity could be improved to 96% ee by using a protein variant containing the single mutation S112G:[54] [Rh(**Biot-1**)COD]$^+$ ⊂ S112G. In contrast, the use of avidin as a scaffold using the same ligand reversed the enantioselectivity to the (*S*)-product (39–44% ee).[53,54] Chemical optimization by introduction of achiral spacers between the biotin anchor and the diphosphine moiety led to the identification of a *meta*-substituted aromatic amino acid spacer (4^{meta}) [Rh(**Biot-**4^{meta}-1)COD]$^+$ ⊂ WT Sav, which also affords (*S*)-reduction products using streptavidin (Fig. 5).[55,65]

Having identified a promising spacer, we proceeded to genetically optimize the artificial metalloenzyme. The best mutants identified in this context had a cationic (or polar) residue at position 112, which suggest that enantioselectivity can be explained by mechanisms reminiscent of Knowles's quadrants (Fig. 6).[15] We hypothesize that the position of the biotinylated ligand, which is largely determined by the spacer, favors one enantioenriched conformation of the rhodium-diphosphine chelate ring. In the quadrant spirit (*i.e.* face-edge array, see Fig. 1C),[15] the resulting orientation of the diphenylphosphine moieties dictates the preferred approach of one prochiral face of the substrate. That the introduction of a spacer with **Biot-**4^{meta}-1 leads to an inversion of the enantioselectivity suggests that the ligand scaffold has inverted its conformation; further, we hypothesize that ionic hydrogen-bonding of a cationic (or polar) group as position 112 with the carboxylate group of the substrate contributes further to favour the production of (*S*)-reduction products. The speculative model

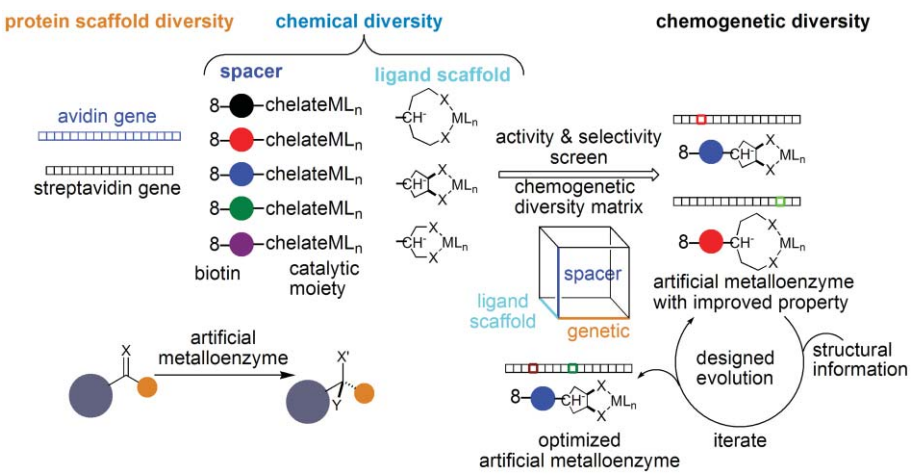

Fig. 4 Designed evolution of an artificial metalloenzyme for asymmetric catalysis based on the biotin-(strept)avidin technology. Starting with a subset of appropriate protein scaffolds and chemically diverse homogeneous catalysts, a chemogenetic diversity matrix can be used to screen for improved characteristics. Designed evolution consists of iterative rounds of screening and selection of the chemogenetic diversity that is introduced, at least partially, according to the structural information available.

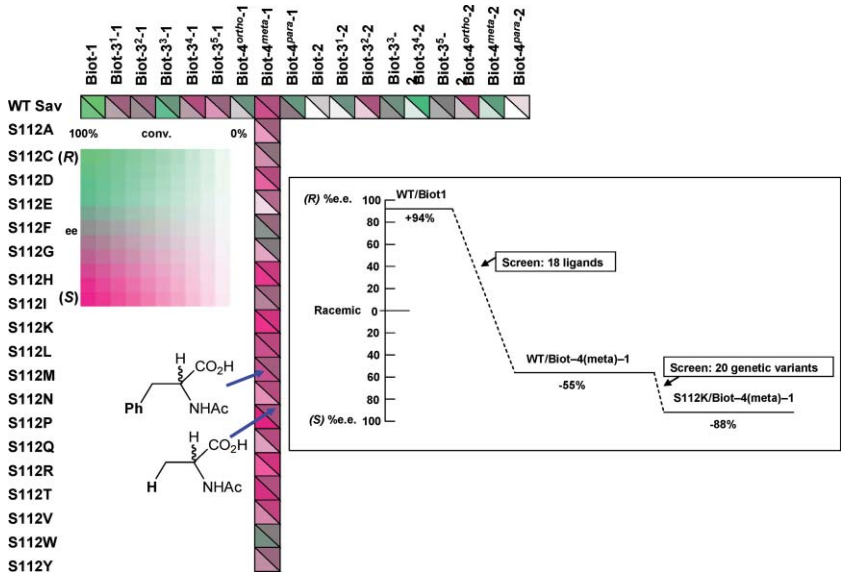

Fig. 5 Fingerprint display of the results for the chemogenetic optimization of the (*S*)-enantioselective reduction of α-acetamidoacrylic acid (top triangle) and α-acetamidocinnamic acid (bottom triangle) in the presence of eighteen biotinylated ligands and twenty streptavidin proteins obtained by saturation mutagenesis at position S112X. Inset: using wild-type streptavidin (WT-SAV) combined with **Biot-1** as a starting point, two steps of the evolution of the artificial enzyme (first step: chemical diversity; second step: genetic diversity) lead to reversal and improvement of enantioselectivity for the hydrogenation of α-acetamidocinnamic acid from 94% ee in favour of (*R*) to 88% ee in favour of (*S*).

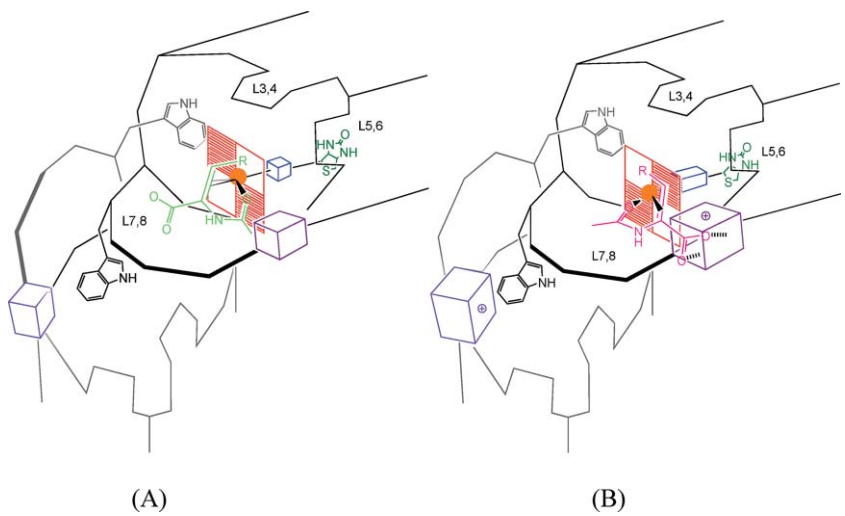

Fig. 6 Schematic representation of the proposed enantiodiscrimination step in the artificial hydrogenases based on the biotin-avidin technology. Streptavidin homo-dimer, emphasizing the loops which make up the biotin binding site (L3,4; L5,6 and L7,8), the critical W120 (hydrophobic lid) provided by the adjacent monomer and the position 112 subjected to saturation mutagenesis (violet cubes). Incorporation of **Biot-spacer-ligand** (green, blue and red respectively) enforces an enantioenriched conformation of the rhodium-diphenylphosphine chelate (see Fig. 1). The quadrants (red squares) depict the steric demand imposed by the ligand, determining the preferred approach of the substrate: green leading to (*R*)-products (A) and red leading to (*S*)-products (B).

presented in Fig. 6 awaits structural confirmation and forms the working hypothesis for future studies. The detailed structural characterisation of our artificial enzymes, at present a major emphasis of our laboratory, is likely to be extremely informative in explaining how the second coordination sphere influences this catalytic reaction.

In addition to the hydrogenation of *N*-acylamido dehydroaminoacids,[54,55,58,65] our group has extended the biotin-(strept)avidin system to carry out transfer hydrogenation[60,61] and oxidation[74] reactions. Moreover, the vast potential for enantioselective transition-metal catalysis has also led us to explore carbon–carbon bond formation, which is difficult to achieve using "biology" alone. These new reactions represent current on-going efforts in our laboratory.

What are the evolutionary advantages of (strept)avidin as a protein host?

Having illustrated in previous sections how the strept(avidin)-biotin system can be exploited for the design of artificial

metalloenzymes, this section explores some of the less-obvious factors that have contributed to the success of these evolutionary studies.

As for natural enzymes with industrial applications, the success of artificial metalloenzymes is dependent on a series of characteristics other than the catalytic performance *per se.* There is a great emphasis in searching for thermostable or solvent-tolerant enzymes for industrial application; on designing artificial enzymes, it will also be advantageous that a robust scaffold is found as a starting point for evolution, which can resist strong selective pressures.

The following are some of the attractive features of using (strept)avidin as scaffolds for artificial enzymes:

Expression

Both avidin[75] and streptavidin[54,76,77] are available as recombinant proteins at relatively high yields (our lab routinely expresses more than 200 mg L^{-1} of *E. coli* culture). Furthermore, both proteins are easy to purify to homogeneity by affinity chromatography with immobilised 2-iminobiotin.

Strong non-covalent binding to biotin

The extreme binding affinity for biotin (K_d around 10^{-15} M) means that the protein–catalyst hybrid complex forms very efficiently and spontaneously. Furthermore, although the affinity for biotinylated catalysts is lower than for biotin, a wide range of biotinylated derivatives are specifically inserted into the biotin-binding site.[78]

Stability

Streptavidin is one of the most thermally-stable proteins identified to date. The biotin-bound tetramer resists more than 110 °C for several minutes.[79] Further, denaturation of the protein requires extreme conditions,[80] such as prolonged exposure to 6 M guanidinium chloride at pH 1.5. Streptavidin is stable at high concentrations of organic solvents, such as 50% ethanol, and to detergents, such as sodium dodecyl sulfate (SDS),[81] leading to the possibility of carrying out reactions in the presence of organic solvents and detergents.

Structure

The crystal structure of many variants of avidin and streptavidin are known;[82] since the biotin-binding amino acids are well-defined, the amino acids closest to the putative active site of the artificial enzymes can be modelled. The "active site" of the artificial enzymes is predicted to be a cleft at the interface between monomers, thus allowing for many interactions of the second coordination sphere.

Mutagenesis

The modelled active site of streptavidin is surrounded by many residues on flexible loops at the surface of the protein whose mutation in most cases does not appear to hinder biotin binding or greatly affect the stability of the protein. Moreover, avidin and streptavidin show high sequence variation (only about 30% sequence identity), whilst still binding biotin effectively. Thus, the protein scaffold is robust and amenable to extensive genetic modification[83] of the second coordination sphere.

Fusion partners and macromolecular complexes

Streptavidin has been used extensively as a fusion partner for other proteins, which opens up the possibility of a "modular construction"[84] of an artificial enzyme, for example by incorporating new domains[85] for specific binding to target molecules. Furthermore, the tetrameric structure of streptavidin can be used for immobilisation or to form supramolecular complexes.

In addition to the above, a general advantage of using a protein scaffold over, for example, molecular imprinting of polymers, is that protein flexibility appears to be crucial in effective catalysis.[86] The fact that extensive "loops" are found close to the active site may be beneficial in evolving streptavidin-based artificial catalysts, even as compared to other protein-based systems that have had limited success, such as catalytic antibodies. For the (strept)avidin artificial metalloenzymes, we reported protein-accelerated catalysis;[56] we speculate that protein flexibility may contribute to this phenomenon.

It is noteworthy that in the structurally-related lipocalin (β-barrel) superfamily of proteins, to which the avidins belong, there is only one protein recognised as an enzyme: prostaglandin D synthase.[87] Although suggestive of a possible evolutionary limitation to this kind of scaffold for catalytic activity, it nevertheless shows that nature has already successfully used this general scaffold for enzyme activity. Therefore, it will be interesting to explore how accurately artificial metalloenzymes based on a (strept)avidin scaffold are able to exploit the beneficial features of natural enzymes.

Some avidins have been reported to have promiscuous[88] pseudocatalytic esterase activity and been suggested as good structural models for the study of enzyme catalysis.[89] Because of their subtle conformational changes and of their complex and dynamic second coordination sphere, we suggest that artificial enzymes such as the ones described here may be much better model systems for the detailed understanding of enzymes than homogeneous catalysts.

The concept of protein space in artificial metalloenzymes

The recent introduction of evolutionary approaches for the design of artificial enzymes, as illustrated above, raises one final hitherto unexplored possibility: that the exploration of protein sequence space[90–92] in artificial enzymes may be quite different to the directed evolution of enzymes. The chemogenetic approach and designed evolution allow great leaps through the fitness landscape that are not possible through directed evolution methods that are commonly used (Fig. 7).[93] For example, changing the metal complex, protein scaffold or a domain in the host can confer drastically new properties (*e.g.* selectivities) by leaping through functionally enriched[94–97] protein space.

Further, enzymes that have been designed by evolution may have intrinsic constraints such that, to achieve a sought level of "fitness" for a trait that has already been partially-selected, requires an initial decrease in fitness and an extensive exploration of protein space. In contrast, the protein scaffold of a hybrid catalyst will have experienced little of the evolutionary pressures that, in enzymes,

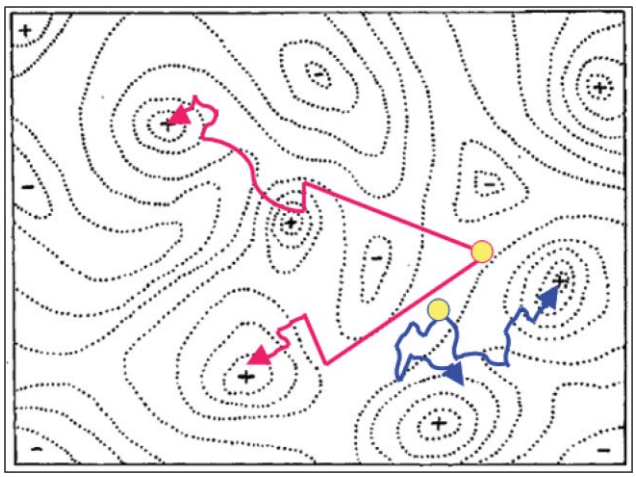

Fig. 7 Diagrammatic representation of a hypothetical landscape of catalyst fitness. Dotted lines: contours with respect to adaptiveness. Yellow circles: starting points of the "wild-type" catalysts. Blue and red lines: pathways of improved fitness of a catalyst *via* directed evolution and *via* designed evolution by chemogenetic optimisation, respectively. Note that two possible pathways are depicted for each type of evolution and that all pathways aim toward maxima ("peaks") represented by plus signs. Directed evolution moves by small incremental steps toward a local maximum, but cannot cross extended regions of local minima (the "valleys" represented by negative signs). By contrast, designed evolution makes great leaps across the landscape and can explore a much wider region of fitness space with fewer steps, including leaping over local minima. Adapted from Wright (1932).[90]

lead to putative structural constraints; thus already largely free of constraints, directed evolution of artificial enzymes may be able to find advantageous mutations through quick "broad exploration" of large sections of protein space. As an example, one can imagine that changing the specificity of a natural enzyme to another, very different, substrate may first involve an initial "opening" of the active site (which evolved due to its natural substrate) that does not change the reactivity of the active site, quickly followed by minor adjustments to confer the new specificity. A hybrid artificial enzyme, however, is largely unconstrained by the possible effect of any mutations on the activity of the enzyme, first, because the rate of activity of the homogeneous catalyst is largely independent of the second coordination sphere; second, the "active site" is already likely to be open and accessible to substrates; third, by starting further away from the "optimum", more protein space can be explored, possibly reaching higher local maxima with small additive effects. In anthropocentric terms, it is better to start from "scratch" than to try to make amends to a system that is not working! Therefore, intuitively, finding improved variants may be easier for hybrid catalysts than for conventional enzymes; it remains to be seen whether this prediction is met in practice.

Conclusion and perspectives

Artificial enzymes at present appear largely restricted to the field of research. But where does the potential of artificial (metallo)enzymes lie? What will be the limitations of their use?

In the first instance, we envisage that hybrid catalysts will provide excellent model systems for the development of new catalysts and for the understanding of reaction mechanisms.

As concerns industrial application of artificial metalloenzymes, their current high cost of development does not warrant their immediate use. Hybrid catalysts may first be viably implemented in cases where cost is low in relation to the high value of the product of the reaction they catalyse, despite their limited turnover numbers. The first practical use of artificial metalloenzymes could be envisaged in very specialized applications in the biomedical field, in the synthesis of specialized and expensive products. In this context, P450-catalysed reactions offer encouraging examples: already some excellent results have been achieved with these difficult and relatively low-turnover, but industrially attractive reactions.[98] We trust that artificial (metallo)enzymes will follow the example of P450 enzymes and be applied, eventually, in the pharmaceutical industry.

Although the "white" biotechnology industries could profit from the practical application of artificial metalloenzymes, for example in the synthesis of new high-value products, the possibility of genetic engineering and the "modular" design of artificial enzymes gives rise to a further intriguing possibility: the future use of artificial (metallo)enzymes in the "red" biotechnology industry, also *in vivo*, for example, to target macromolecules such as DNA for cancer therapy.[99] Already, (strept)avidin has been considered as a delivery vehicle of diagnostic radioactive metals *in vivo*;[100] protein variants of (strept)avidin with lower antigenicity are being sought for repeated administration in humans[101] and the incorporation of targeting domains for entry into cells has started to be explored.[102]

In conclusion, artificial metalloenzymes have great potential for application in very diverse fields of the white and red biotechnology industries; both biology and chemistry will contribute to their development.

What are the perspectives for artificial enzymes? One hundred years after Buchner's Nobel Prize for a discovery that "fused" chemistry and biology, we are still only just beginning to explore the potential of hybrid artificial enzymes; in the next few years, we look forward to seeing great developments in this broad, interdisciplinary field of biomolecular and organic chemistry.

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