Directed evolution of hybrid enzymes: Evolving enantioselectivity of an achiral Rh-complex anchored to a protein

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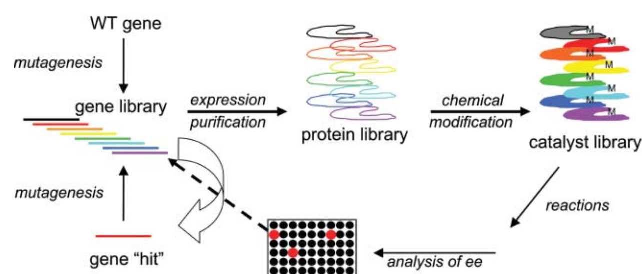
The concept of utilizing the methods of directed evolution for tuning the enantioselectivity of synthetic achiral metal–ligand centers anchored to proteins has been implemented experimentally for the first time.

Directed evolution is a powerful method for enhancing the stability, activity and/or selectivity of enzymes.[1] This approach to protein engineering comprises repeating cycles of appropriate random gene mutagenesis, expression of mutant enzymes and high-throughput screening (or selection) for a given catalytic property. In 1997 we showed that this strategy can be used to enhance the enantioselectivity of enzymes catalyzing the transformation of unnatural substrates;[2] later reversal of enantioselectivity was demonstrated.[3] This novel approach to asymmetric catalysis has since been applied to many different types of enzymes.[4] However, enzymes are incapable of catalyzing many if not most reactions that chemists have invented using transition metals.[5] Therefore, we have previously proposed the use of directed evolution as a means to tune hybrid catalysts.[6] This concept is based on the well-known fact that it is possible to chemically modify a protein by covalent or non-covalent anchoring of a metal–ligand moiety to produce an individual potential catalyst.[7] In our approach a library of protein mutants is first produced by random mutagenesis and then subjected to an appropriate post-translational chemical modification. This provides a pool of mutant hybrid catalysts, each having a synthetic metal center in a different protein environment (Scheme 1).

Subsequent screening for enantioselectivity leads to the identification of an improved mutant (hit), and the mutant gene encoding the respective protein can then be used as a template for another cycle, thereby imparting evolutionary pressure on the system (Scheme 1). As in the case of directed evolution of enzymes,[1] multiple cycles are possible which may be needed.[6]



Scheme 1 Directed evolution of hybrid catalysts in which metal (M)–ligand is anchored non-covalently.

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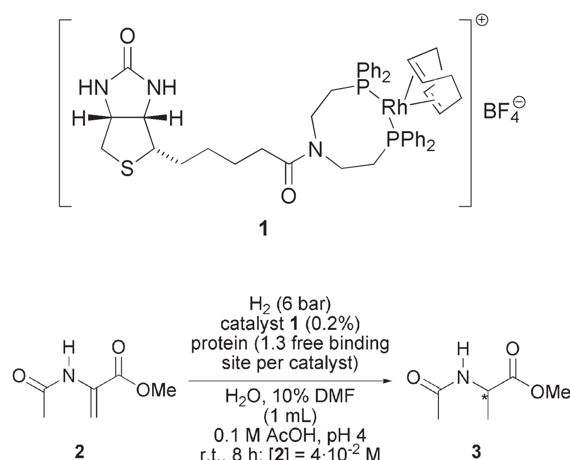
The experimental implementation of this concept is challenging because several non-trivial prerequisites need to be fulfilled: (1) The host protein should be robust; (2) An efficient expression system, delivering sufficient protein in miniaturized and parallelized form has to be available; (3) The host protein mutants need to be purified in parallel; (4) Chemical modification with introduction of a metal–ligand entity should be essentially quantitative; (5) A high-throughput screening system has to be available for a given reaction of interest.[8]

Parallel to efforts employing covalent chemical modification,[6] we proposed in 2002 the non-covalent variant as an alternative,[6b] specifically the use of the Whitesides system[9] based on the strong interaction of a chemically modified biotin with the host protein avidin. In that system biotin is first attached covalently to an achiral diphosphine–Rh complex *via* a spacer to form complex **1** which then binds with high affinity to avidin. The wild-type (WT) avidin–**1** complex was used as a single catalyst in the Rh-catalyzed hydrogenation of α-acetamido-acrylic acid, the resulting ee ranging between 33 and 44% depending upon the conditions used.[9a] Ward has used chemical tuning and rational protein design to improve the enantioselectivity significantly in the same reaction,[10] which is fundamentally different from our Darwinian approach. In the present project we likewise employed the biotinylated diphosphine–Rh complex **1**, but chose to use the esterified substrate **2** because this facilitates parallel analysis by gas chromatography (Scheme 2). The reaction mixtures can be extracted with ethyl acetate in a parallel manner, whereas the acid of the Whitesides–Ward system is accessible efficiently only by continuous extraction.



Scheme 2 Rh-catalyzed hydrogenation of α-acetamido-acrylic acid ester.

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Although several expression systems for avidin have been described, production of eukaryotic proteins is both time consuming and low yielding. We therefore turned to streptavidin, a genetically unrelated bacterial protein which also binds biotin with high affinity. Several expression systems for streptavidin have been described,[11] and some of them were compared. Unfortunately, problems arose with the expression level and purification in parallel form. The best solution for our needs is based on pET11b-sav.[12] This construct encodes 12 residues of T7-tag followed by Asp and Gln and residues 15 to 159 of the mature streptavidin. *Esherichia coli* strain BL21(DE3) transformed with this plasmid and grown in Studier's auto-induction media,[13] ZYP5052, requires less monitoring than conventional induction with IPTG at the mid-log phase and thus allows multiple unattended overnight cultures. This adaptation was crucial in simplifying the screening task. However, the system is still not fully suited for screening thousands of mutants since it requires a 150 mL culture scale to provide a sufficient amount of streptavidin. Typically we used 1.04×10^{-7} mol of binding site, which is equivalent to ~1.7 mg protein (based on a MW of 16.5 kDa per monomer and expecting 3.8–3.9 free binding sites per tetramer as obtained for the WT). However, when lower amounts of streptavidin were obtained for a given mutant, the culture scale was increased by up to five-fold and/or the amount of hybrid catalyst used in the reaction was decreased to 0.1%. Titrated mutant streptavidins were transferred into glass vessels of an in-house adapted reactor block[14] for the Chemspeed Accelerator™ SLT 100 Synthesizer.

We therefore settled for only a few hundred mutants in each mutagenesis experiment and proceeded with directed evolution on a "small scale" which in fact was still labor-intensive. In an initial experiment, the WT-streptavidin–**1** was found to be a poor catalyst in the hydrogenation of **2**, leading to an ee of only 23% in favour of (*R*)-**3**. Rather than targeting the whole protein for amino acid substitution by error-prone PCR,[1] we applied CASTing (CAST = Combinatorial Active-site Saturation Test),[15] in which appropriate amino acid sites next to the binding pocket are randomized by saturation mutagenesis, specifically in an iterative manner.[16] In the present case an X-ray structure of the conjugate has not been reported. Therefore, the CAST sites for amino acid randomization were chosen on the basis of modeling the biotinylated Rh-complex **1** into the X-ray structure of streptavidin–biotin.[17] Fig. 1 shows an excerpt of the modeled structure and the amino acid sites that appeared to be appropriate for CAST experiments.

The positions that we considered for saturation mutagenesis can be classified into two categories. The first are "close" positions, for example Asn49, Leu110, Ser112 and Leu124, which are located about 4 to 6 Å away from the Rh(I) of the two calculated major conformers. These could influence directly the conformation of the catalyst or catalyst–substrate complex. The second type of positions are "distal": Glu51, Tyr54, Trp79, Asn81, Arg84, Asn85, His87, which are located further away from Rh(I). These "second sphere" CAST-positions could influence the structure of the enzyme as a whole because they are involved in hydrogen bonding between secondary elements.

We started saturation mutagenesis at positions 110, 112, and 124 using the QuikChange method (Stratagene) and pET11b-sav.[12] In each saturation experiment about 200–300 clones (oversampling for >95% coverage) were harvested and tested.

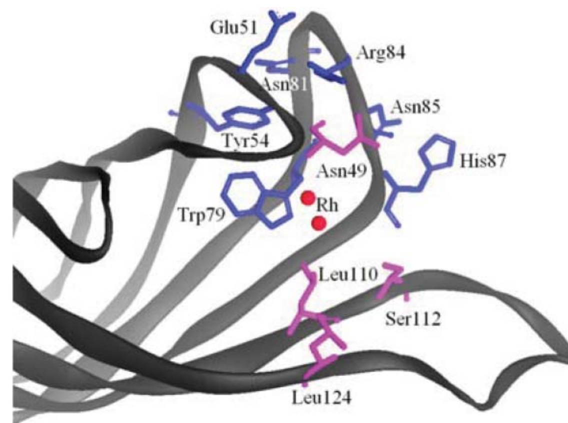
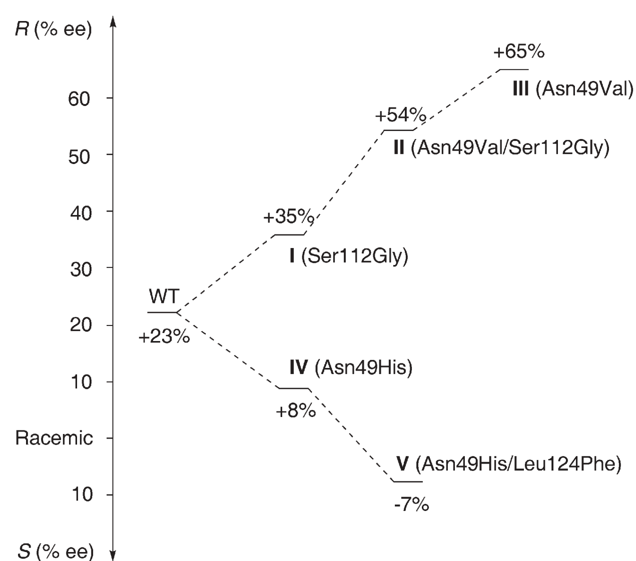


Fig. 1 Selected close (purple) and distal (blue) sites from Rh(I) centers (red) of two important calculated conformers of the complex.

In some cases protein variants were obtained in amounts too small for fulfilling the experimental prerequisites for reproducible hydrogenation, and these were not considered for further study. Nevertheless, a few mutants showing enantioselectivity different from the WT were observed in a reproducible manner, the best variant **I** leading to 35% ee (*R*). It is characterized by mutation Ser112Gly. However, a single round of saturation mutagenesis does not yet constitute an evolutionary process. Therefore, iterative CASTing was performed using the gene that encodes mutant **I** and saturating at position 49. This led to a double mutant **II** having mutations Asn49His/Ser112Gly and showing an ee-value of 54% (*R*) in the model reaction (Scheme 3). Finally, a third-generation saturation experiment was performed using the gene which encodes mutant **II** and focusing once more on position 112. This experiment was designed to test whether glycine at position 112 is really the best choice when combining with histidine at position 49. An improved mutant **III** was identified leading to an ee of 65% (*R*) in which, surprisingly, the original mutation Ser112Gly was



Scheme 3 Directed evolution of hybrid catalysts comprising streptavidin–**1**, the Rh-catalyzed hydrogenation **2** → **3** serving as the model reaction (40–90% yield).

reverted back to serine (Scheme 3). Thus, the best variant of this study is characterized by a single mutation (Asn49Val). The increase in selectivity corresponds to about $\Delta\Delta G^{\neq}$ = 2.7 kJ $\times$ mol^{-1}. Mutagenesis experiments at positions 51, 54, 79, 81, 84, 85 and 87 were not successful because no soluble protein was obtained. In contrast, saturation mutagenesis at position 49 on WT template led directly to mutants **III** and **IV**. The latter is characterized by Asn49His. This variant has lower enantioselectivity than the WT (ee = 8% (*R*)), suggesting the possibility of inverting stereoselectivity. The plasmid encoding variant **IV** was utilized as a template for saturation mutagenesis. Indeed, upon focusing on position 124, mutant **V** (Asn49His/Leu124Phe) was identified which is (*S*)-selective, although not by a great degree (ee = 7%). The essential evolutionary steps are summarized in Scheme 3. We observed no clear relationship between enantioselectivity and rate, although differences in activity were observed.

In summary, our work demonstrates that it is possible to apply the methods of directed evolution to increase and/or to invert enantioselectivity of a hybrid catalyst composed of a synthetic achiral transition metal catalyst anchored to a host protein. Due to the problems associated with the expression system, the full potential of this novel approach to asymmetric catalysis was not tested in the present system. However, proof-of-principle has been achieved for the first time, providing incentive for designing and testing other systems.[18]

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