Artificial Metalloenzymes

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OsO_4·Streptavidin: A Tunable Hybrid Catalyst for the Enantioselective *cis*-Dihydroxylation of Olefins**

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Enzymatic and homogeneous catalysis have evolved independently to address the challenges in the synthesis of enantiopure products. With the aim of complementing these fields, artificial metalloenzymes, which combine the structural diversity of biocatalysts with the wealth of metal-catalyzed reactions, have attracted increasing attention.[1] In homogeneous catalysis the *cis*-selective, OsO_4-dependent asymmetric dihydroxylation (AD) of olefins ranks among the most powerful methods for the synthesis of vicinal diols. Ligands for homogeneous catalysis have been largely developed by Sharpless and co-workers, and are, with few exceptions, almost exclusively based on quinidine or quinine derivatives.[2] Although most classes of prochiral olefins are dihydroxylated with good activity and selectivity, the *cis*-substituted olefins are problematic. Nature relies on non-heme iron dioxygenases such as naphthalene dioxygenase (NDO) to perform a related reaction. These enzymes display broad substrate scope.[3] It is believed that both the OsO_4- and NDO-catalyzed dihydroxylations proceed by an outer sphere [3+2] mechanism in which the substrate is not bound to the metal in the transition state (Figure 1).[2a,f,3c]

Considering a biomimetic approach, we hypothesized that anchoring a catalytically competent Os^{VIII} center within a protein might afford an artificial metalloenzyme for the AD of olefins. Encouraged by a report by Kokubo et al.,[2d] we set out to screen various proteins and to test whether the resulting dihydroxylases could be optimized by genetic means.

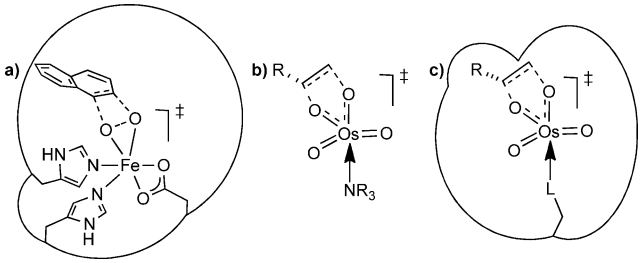
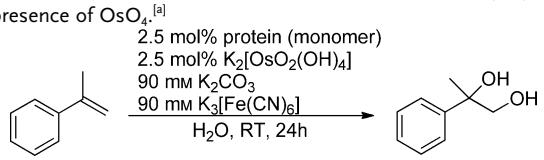


Figure 1. Postulated transition-state structure for the dihydroxylation of olefins: a) for the naphthalene dioxygenase; b) for the osmium-catalyzed AD of prochiral olefins; and c) for an artificial *cis*-dihydroxylase resulting from anchoring of OsO_4 within a host protein.

Five proteins were evaluated as hosts for the AD of α-methylstyrene: Wild-type streptavidin (SAV) clearly performed best. In contrast, bovine serum albumin (BSA) yielded the opposite enantiomer, albeit with a low turnover number (Table 1). In view of the size of the proteins (66 kDa for BSA and 16 kDa for the SAV monomer), the difficult recombinant production of BSA,[4] and the number of

Table 1: Identification of a suitable host for the AD of α-methylstyrene in the presence of OsO_4.[a]



Entry	Protein	*ee* [%][b]	TON[c]
1[d]	avidin	2 (*R*)	16
2	BSA	77 (*S*)	4
3[e]	lysozyme	5 (*S*)	6
4[f]	human carbonic anhydrase II	25 (*S*)	<1
5[g]	SAV	95 (*R*)	27

[a] Reactions were carried out with 6 μmol substrate in a total reaction volume of 0.2 mL. [b] *ee* value determined by HPLC on a chiral stationary phase; absolute configuration assigned by comparison with literature data. [c] Determined by reverse-phase HPLC with internal standard. TON = mol product per mol $K_2[OsO_2(OH)_4]$. [d] Some precipitation was observed at the end of the run. [e] Immediate precipitation occurred upon addition of $K_2CO_3/K_3[Fe(CN)_6]/K_2[OsO_2(OH)_4]$ stock solution to the protein stock solution. [f] Protein was not fully soluble. [g] Average of two independent runs.

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histidine residues in BSA (17 His for BSA vs. 2 His per SAV monomer), SAV was selected as the host for further studies.

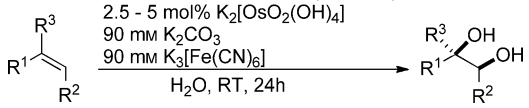
Next, a range of substrates was subjected to AD in the presence of SAV. With the exception of indene and precocene I, all the substrates produced the same enantiomer as with the quinidine scaffold in the Sharpless AD (i.e. AD-mix-β).[2a,h–l] Although some substrates were converted with a low to moderate *ee* value, both *cis*-β-methylstyrene and α-methylstyrene were dihydroxylated with good selectivity (Table 2). The expected *syn* specificity of the reaction was demonstrated unambiguously (i.e. 1% or less *anti* product detected) by comparison of the dihydroxylation products of *cis*-β-methylstyrene and *trans*-β-methylstyrene (see Supporting Information).

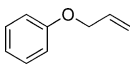
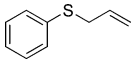
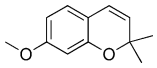
To investigate the fate of SAV under the oxidizing conditions, the reaction mixture was subjected to SDS-PAGE after catalysis, and a remaining biotin-binding capability was revealed by biotin-4-fluorescein staining of the gel. As the *ee* value remained constant over the course of the reaction, we concluded that any decomposition pathway of the catalyst leads to species with strongly reduced activity (see Supporting Information).

Addition of 1.05 equivalents of biotin per SAV monomer afforded a nearly racemic product for the AD of α-methylstyrene. This finding suggests that the catalytically active Os moiety is located in the vicinity of the biotin-binding pocket (Figure 2b and 2c). As the carboxylate D128 ··· HN_{urea} hydrogen bond of biotin is a key interaction for high biotin·SAV affinity,[8] SAV D128A was tested in the AD. α-Methylstyrene was converted by the OsO_4·SAV D128A catalyst with significantly diminished selectivity.[9] The AD of allyl phenyl ether in the presence of SAV D128A afforded the opposite enantiomer than with SAV and allyl phenyl sulfide was converted with remarkable selectivity. In contrast, the conservative mutation SAV D128E, which extends the side chain of residue 128 by a methylene group, led to less dramatic changes in the enantioselectivity (Table 2).

Further insight on the location of the catalytically active Os species was gathered by mutation of further residues located close to the biotin-binding site. Inspection of the SAV structure revealed that the L-7,8 loops (residues 113–121, Figure 2b) of two neighboring monomers line the biotin-binding pocket.[10] Single point mutants at position S112, K121, and L124 were combined with various amounts of $K_2[OsO_2(OH)_4]$ and tested in AD.[11] Mutation at position SAV S112 proved most effective for fine-tuning purposes. Both SAV S112Y and SAV S112M led to an improvement of selectivity in many cases. α-Methylstyrene was converted with 75% *ee* (*R*) with SAV S112Y and 97% *ee* (*R*) with SAV S112M. Allyl phenyl sulfide, which gave nearly racemic product with SAV, was converted with 71% *ee* (*S*) with SAV S112Y. The best result for the AD of 1,2-dihydronaphthalene was obtained with SAV S112M (41% *ee*, 1*R*,2*S*). The conservative mutation SAV S112T, which introduces an additional methyl group to the amino acid side chain of residue 112, proved best for the dihydroxylation of precocene (68% *ee*, 3*R*,4*R*) and of *cis*-β-methylstyrene (92% *ee* 1*R*,2*S*), Table 2).

Table 2: Genetic optimization of the performance of artificial AD.[a]



Entry	Olefin	SAV mutant (mol% Os)[b]	*ee* [%][c]	TON[d]	Ref. *ee* [%] [Ref.]
1		WT (2.5)	40 (*S*)	13	
2		S112Y (5.0)	82 (*S*)	14	88
3		D128A (2.5)	77 (*R*)	21	[2h]
4		D128E (2.5)	0	9	
5		WT (2.5)	2 (*R*)	4	
6		S112Y (5.0)	71 (*S*)	7	61
7		D128A (2.5)	71 (*R*)	10	[2i]
8		D128E (2.5)	12 (*R*)	5	
9		WT (2.5)	30 (1*R*,2*S*)	13	56 [2j]
10		S112Y (5.0)	7 (1*S*,2*R*)	12	
11		S112M (5.0)	41 (1*R*,2*S*)	12	
12		WT (2.5)	90 (1*R*,2*S*)	26	
13		S112Y (5.0)	45 (1*R*,2*S*)	11	72
14		S112T (2.5)	92 (1*R*,2*S*)	16	[2j]
15[e]		S112T (2.5)	91 (1*R*,2*S*)	≥16	
16		WT (2.5)	5 (1*S*,2*R*)	12	53 [2k]
17		S112Y (5.0)	16 (1*R*,2*S*)	12	
18		D128A (2.5)	45 (1*R*,2*S*)	13	
19		WT (2.5)	62 (3*R*,4*R*)	6	67 [2l]
20		S112Y (5.0)	26 (3*R*,4*R*)	5	
21		S112T (2.5)	68 (3*R*,4*R*)	6	
22		WT (2.5)	95 (*R*)	27	
23		K121N (5.0)	24 (*R*)	8	
24		L124G (5.0)	16 (*R*)	11	
25		L124K (2.5)	89 (*R*)	18	
26		S112Y (5.0)	75 (*R*)	15	99
27		S112M (5.0)	97 (*R*)	16	[2n]
28		D128A (2.5)	53 (*R*)	22	
29		D128E (2.5)	80 (*R*)	21	
30[f]		WT (2.5) + biotin	9 (*R*)	13	

[a] Results are the average of two independent runs, see Table 1 and Supporting Information for experimental details. [b] The ideal osmate loading was determined for each mutant (see Supporting Information and Ref. [7]). [c] *ee* determined by HPLC on a chiral stationary phase; absolute configuration assigned by comparison with literature data. [d] Determined by reverse-phase HPLC with internal standard. TON = mol product/mol $K_2[OsO_2(OH)_4]$. [e] Carried out on a 120 μmol scale; TON is based on yield of isolated product. [f] 1.05 equivalents of D-biotin were added relative to protein monomer.

The system performed well on a range of challenging substrates. Noteworthy results were obtained for allyl phenyl sulfide (Table 2, entries 6, and 7) and *cis*-β-methylstyrene (Table 2, entry 14) and are the highest *ee* values ever reported for these substrates to the best of our knowledge.

For other demanding *cis*-substituted substrates, such as 1,2-dihydronaphtalene and indene, the enantioselectivities could be significantly improved by site-directed mutagenesis

(Table 2, entries 11 and 18). For the AD of precocene, the *ee* value obtained with SAV S112T matched the best result obtained with quinidine-derived systems.

Importantly, exchange of the lysine side chain at position 121 resulted in low levels of stereoselectivity: the highest *ee* value observed for any SAV K121 mutant/substrate combination was 24% (*R*) (α-methylstyrene; Table 2, entry 23). Mutating the leucine L124 had a less pronounced deleterious (or neutral in one case) effect on selectivity.

With the aim of gaining structural insight on the location of the Os center, SAV crystals were grown (at pH 4.0 or 7.3) and subsequently soaked in a solution of $K_2[OsO_2(OH)_4]$. The resulting crystals were subjected to X-ray analysis (see the Supporting Information).

Soaking crystals, obtained at pH 7.3, resulted in anomalous scattering density (modeled with an Os atom), which was detected in the proximity of four residues: N^{ζ} of K80, N^{ε} of H87, N^{ε} of H127 and N^{ζ} of K132 (Figure 2a). The intrinsic packing disorder, however, did not allow full refinement but yielded precious information on potential Os-binding sites. In contrast, the X-ray data of the crystals obtained at pH 4.0, yielded a well-behaved structure with a single Os binding site close to N^{ε} of the H127 side chain (see the Supporting Information).

The three osmium binding sites identified by X-ray crystallography located in the proximity of the biotin binding site (e.g. H87, H127 and K80) were subjected to site-directed mutagenesis. Catalysis in the presence of single mutants SAV H87A, SAV H127A, and SAV K80G gave comparable results to SAV in the AD of α-methylstyrene, (lowest *ee* 89% (*R*) with H87A; see Table S1 in the Supporting Information).[6] Although the structural data did not allow identification of the actual catalytic site, it suggests that not all of the osmium is catalytically active, which accounts for the modest TONs (up to 27 turnovers per added osmium).[2m]

In summary, we have demonstrated that high selectivity for challenging substrates can be obtained with an artificial AD based on OsO_4·SAV. Importantly, it was shown that the hybrid catalyst offers vast opportunities for genetic optimization: Single point mutations can lead to a significant increase in enantioselectivity and even to an inversion of enantiopreference. As for several natural mono- and dioxygenases, this artificial metalloenzyme suffers from modest TONs combined with a high molecular weight. Current efforts are thus aimed at increasing the TON by identifying the location and the kinetic profile of the catalytically competent osmium species and testing other challenging substrates, including tri- and tetrasubstituted olefins. Finally, from a biomimetic perspective, combination with a flavin-coupled NMO-recycling system with H_2O_2 as the stoichiometric oxidant would be of great interest.[2n,o]

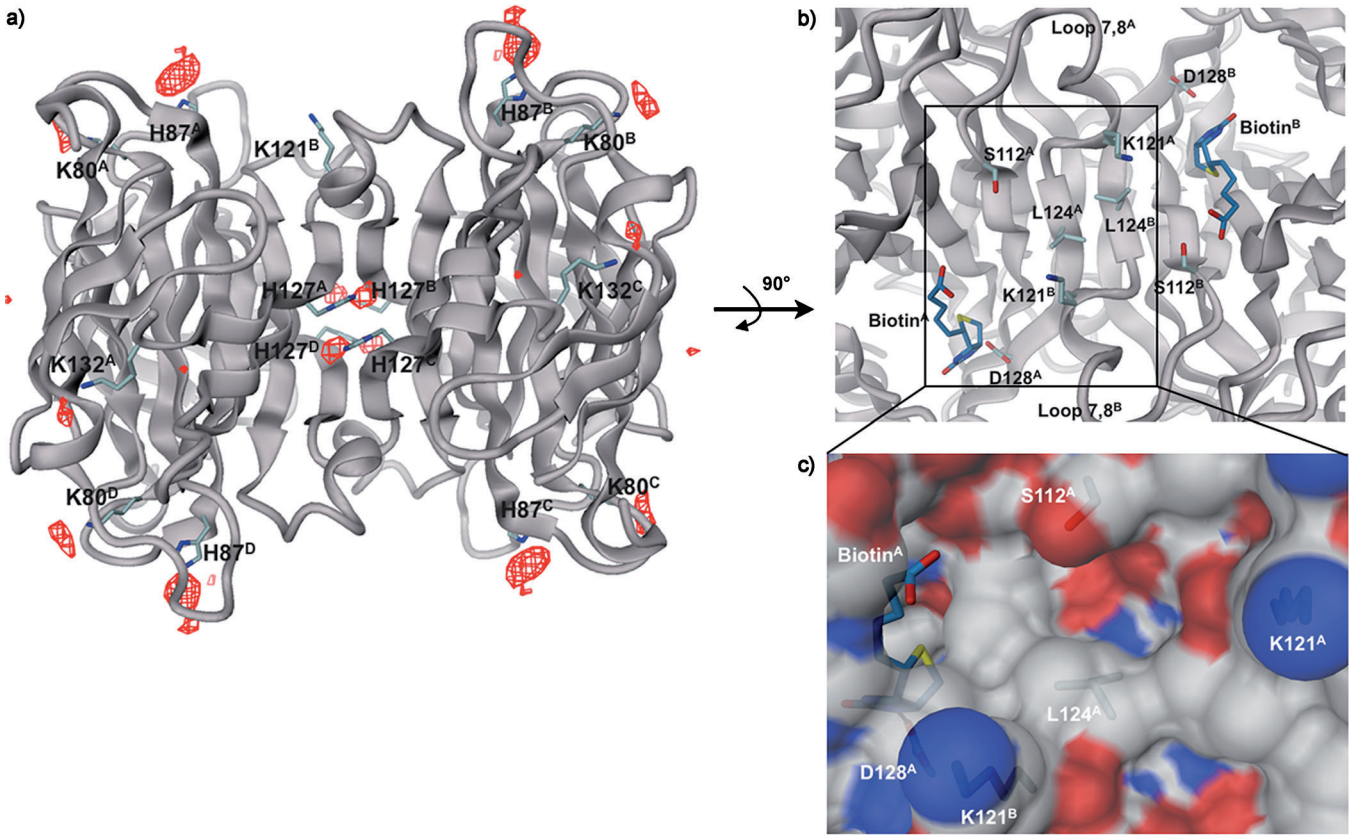


Figure 2. Crystal structure of SAV soaked in $K_2[OsO_2(OH)_4]$ at pH 7.3: a) ribbon representation of SAV tetramer highlighting positions of strong anomalous difference density (in red, contoured at 4σ); b) close-up view of the biotin-binding pocket (from PDB 1stp) highlighting the residues and biotin (stick representation) which strongly influence catalysis; c) surface representation of close-up view.

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[6] Although the effect of mutating K132 has not yet been investigated, the results obtained with mutations at other sites (namely S112, K121, L124, and D128) render the involvement of this amino acid side chain unlikely.

[7] The performance of mutants/batches used in this study was first evaluated with various ratios of osmate to protein to determine the loading that led to the highest HPLC yield without erosion of enantioselectivity; see the Supporting Information for details.

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[10] The residue K121 is significantly closer to residue D128 of the adjacent monomer in the tetrameric structure of SAV than to the D128 on the same oligopeptide chain (12.67 vs. 20.70 Å for C_α).

[11] After an initial screening of a wider range of SAV mutants with styrene, five SAV S112X mutants (S112N, S112A, S112T, S112M, S112Y), four SAV K121X (K121A, K121F, K121N, K121H), and four SAV L124X mutants (L124G, L124K, L124N, L124Y) were selected for further study.

