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Artificial Metalloenzyme for Enantioselective Sulfoxidation Based on Vanadyl-Loaded Streptavidin

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Abstract: Nature's catalysts are specifically evolved to carry out efficient and selective reactions. Recent developments in biotechnology have allowed the rapid optimization of existing enzymes for enantioselective processes. However, the ex nihilo creation of catalytic activity from a noncatalytic protein scaffold remains very challenging. Herein, we describe the creation of an artificial enzyme upon incorporation of a vanadyl ion into the biotin-binding pocket of streptavidin, a protein devoid of catalytic activity. The resulting artificial metalloenzyme catalyzes the enantioselective oxidation of prochiral sulfides with good enantioselectivities both for dialkyl and alkyl-aryl substrates (up to 93% enantiomeric excess). Electron paragmagnetic resonance spectroscopy, chemical modification, and mutagenesis studies suggest that the vanadyl ion is located within the biotin-binding pocket and interacts only via second coordination sphere contacts with streptavidin.

Introduction

The affinity of biotin for streptavidin ranks among the strongest noncovalent interactions found in nature ($K_a \sim 10^{13}$ M^{-1}).[1–3] To achieve such unrivaled affinities, both hydrogen-bonding and hydrophobic interactions are combined to provide an exquisitely tailored biotin binding site.[4–7] This deep cavity can bind, albeit with significantly reduced affinity, a variety of ligands, including HABA, ANS, different oligopeptides, etc.[8–11] In the context of artificial metalloenzymes, we reasoned that a catalytically active small polar coordination compound may interact with the biotin-binding residues via hydrogen bonds. In addition, the presence of hydrophobic residues may favor substrate accumulation within the cavity, resulting in increased turnover rates for the catalyzed reaction. To test the validity of this concept, and inspired by the use of vanadium in biocatalytic oxidations,[12] we selected the vanadyl-catalyzed sulfoxidation of prochiral substrates (Scheme 1).

In recent years, there has been an increasing interest in the creation of artificial metalloenzymes for enantioselective catalysis. With this goal in mind, covalent, supramolecular as well as dative anchoring strategies have been used to ensure the localization of a catalytically active moiety within a chiral macromolecule (protein or DNA) scaffold. The enantioselective reactions implemented thus far include ester hydrolysis,[13] dihydroxylation,[14] epoxidation,[15,16] sulfoxidation,[17–21] hydrogenation,[22–30]

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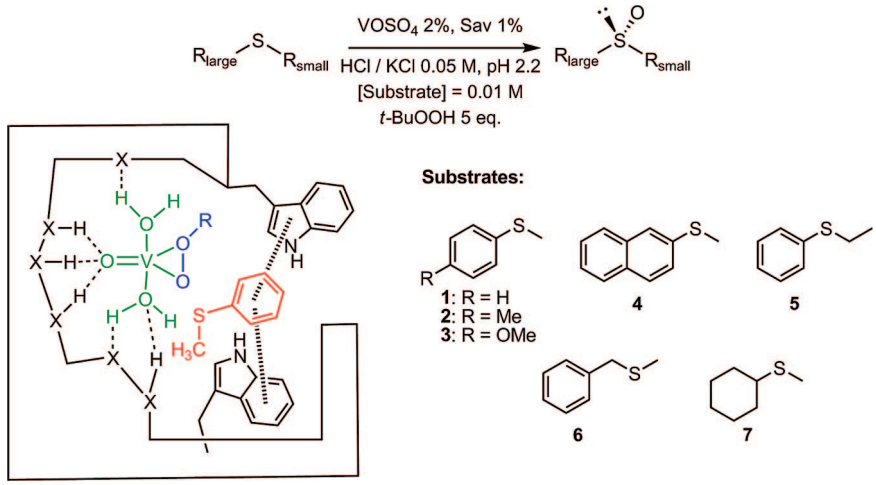
Scheme 1. Vanadium-Dependent Artificial Peroxidase for Enantioselective Sulfoxidation Reactions



transfer hydrogenation,[31–33] allylic alkylation,[34] and Diels–Alder[35–37] and Michael additions.[38] In addition, artificial enzymes devoid of metal cofactors have been reported, for example, based on aspartate-containing peptides,[39] bovine serum albumin (BSA),[40–43] or chymotrypsin[44] as protein templates.[45]

Results and Discussion

With the aim of identifying suitable reaction conditions, we selected thioanisole **1** as a model substrate for the enantioselective sulfoxidation by a vanadyl source in conjunction with streptavidin (abbreviated $[VO]^{2+} \subset$ WT Sav hereafter). To maximize the incorporation of $[VO]^{2+}$ inside the binding pocket of tetrameric streptavidin (which has four binding pockets per tetramer), an excess of streptavidin binding sites was employed, so that two binding sites were available per molecule of $VOSO_4$. When a stoichiometric amount of binding sites versus vanadium was used, comparable conversions but slightly lower selectivities were obtained. In the absence of vanadium, very little conversion was observed, while in the absence of Sav, the activity of the catalyst was only modest (Table 1, entries 1 and 2). Variation of the initial pH and of the oxidizing agent revealed that an HCl/KCl buffer (0.05 M) at pH = 2.2 combined with *t*-BuOOH (5 equiv versus substrate) worked best in terms of both activity and selectivity, while the vanadyl source had no influence on the reaction outcome (Table 1, entries 3–8). On the basis of these results, and in consideration of the speciation of vanadyl ions in acidic media,[46] we suggest that the vanadium species interacting with streptavidin is the pentahydrated vanadyl ion, $[VO(H_2O)_5]^{2+}$. Under optimized conditions with 2 mol %

Table 1. Selected Results for the $[VO]^{2+} \subset$ WT Sav-Catalyzed Oxidation of Sulfides[a]

entry	protein	vanadium source	substrate	oxidant	conv (%)	ee[b] (%)
1	WT Sav		**1**	*t*-BuOOH	7	4
2		$VOSO_4$	**1**	*t*-BuOOH	55	0
3	WT Sav	$VOSO_4$	**1**	*t*-BuOOH	94	46
4	WT Sav	$VO(acac)_2$	**1**	*t*-BuOOH	99	46
5	WT Sav	$VO(OiPr)_3$	**1**	*t*-BuOOH	99	46
6	WT Sav	Na_3VO_4	**1**	*t*-BuOOH	92	46
7	WT Sav	$VOSO_4$	**1**	HOOH	39	0
8	WT Sav	$VOSO_4$	**1**	cumylOOH	82	−15[c]
9	WT Sav	$VOSO_4$	**2**	*t*-BuOOH	96	87
10	WT Sav	$VOSO_4$	**3**	*t*-BuOOH	quant	90
11	WT Sav	$VOSO_4$	**4**	*t*-BuOOH	53	93
12	WT Sav	$VOSO_4$	**5**	*t*-BuOOH	96	90
13	WT Sav	$VOSO_4$	**6**	*t*-BuOOH	quant	73
14	WT Sav	$VOSO_4$	**7**	*t*-BuOOH	61	86
15	WT Sav + biotin (4.25 equiv)	$VOSO_4$	**3**	*t*-BuOOH	96	0
16	D128A Sav	$VOSO_4$	**3**	*t*-BuOOH	97	0
17	avidin	$VOSO_4$	**3**	*t*-BuOOH	78	7
18	BSA	$VOSO_4$	**3**	*t*-BuOOH	quant	0
19	Aviloop	$VOSO_4$	**1**	*t*-BuOOH	quant	60
20	Aviloop	$VOSO_4$	**7**	*t*-BuOOH	54	90

[a] All catalytic runs were performed at room temperature in 0.05 M KCl/HCl buffer at pH 2.2, with 0.0001 M WT Sav, 0.0002 M vanadium, 0.01 M sulfide, and 0.05 M oxidant. [b] *R*-configuration assigned according to ref 47 except as noted. [c] *S*-configuration assigned according to ref 47.

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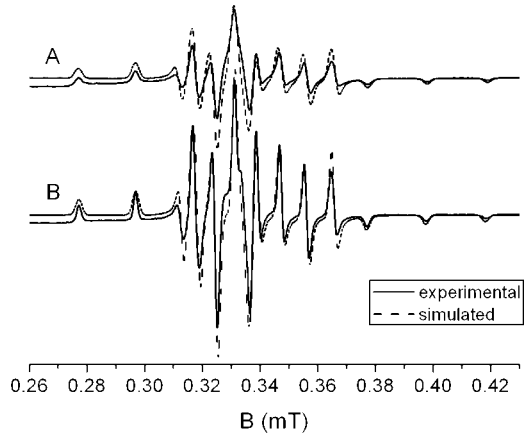

Figure 1. Frozen aqueous solution X-band EPR spectra (—) of (A) $VOSO_4$ and (B) $VOSO_4 \subset$ Sav in a buffer solution at pH = 2.2. $[VOSO_4] = 0.2$ mM (A, B) and [Sav] = 0.1 mM (B). Powder simulation spectra (---) have been calculated with the following EPR spin Hamiltonian parameters: (A) $g_{\|} = 1.932$ cm^{-1}, $g_{\perp} = 1.978$ cm^{-1}, $A_{\|} = 183$ cm^{-1}, $A_{\perp} = 70 \times 10^{-4}$ cm^{-1}, $W_{\|} = 22$ G, and $W_{\perp} = 19$ G; (B) $g_{\|} = 1.933$ cm^{-1}, $g_{\perp} = 1.976$ cm^{-1}, $A_{\|} = 182$ cm^{-1}, $A_{\perp} = 69 \times 10^{-4}$ cm^{-1}, $W_{\|} = 25$ G, and $W_{\perp} = 25$ G.

vanadium and 1 mol % tetrameric streptavidin, the sulfoxidation of thioanisole **1** afforded the corresponding sulfoxide in 94% conversion and 46% ee (*R*) (Table 1, entry 3).

Next, we screened prochiral sulfides **2**−**7** (Scheme 1), which are typical substrates for homogeneous sulfoxidation reactions.[47] For all substrates, very little overoxidation to the corresponding sulfone could be detected by HPLC (<2%). It is interesting to note that increasing the steric bulk of the aromatic moiety of the sulfide leads to an increase in selectivity: up to 93% ee for the sulfoxidation of methyl-2-naphthylsulfide **4** (Table 1, entries 9−12). Oxidation of dialkylsulfides produced the corresponding sulfoxides with reasonable enantioselectivity (up to 86% ee; Table 1, entries 13 and 14).

In order to test whether the vanadyl moiety is incorporated in the biotin-binding pocket, 4.25 equiv of biotin versus tetrameric Sav were added to the reaction mixture. Under these conditions, the sulfoxidation of 4-methoxythioanisole **3** afforded racemic product (Table 1, entry 15). Most interestingly, the presence of biotin-free streptavidin contributes to accelerate the sulfoxidation rate $\{v_{init}([VO]^{2+} \subset \text{WT Sav})/v_{init}([VO]^{2+}) = 3\}$. In contrast, biotin-loaded streptavidin accelerates the rate of the $[VO]^{2+}$-catalyzed sulfoxidation very little $\{v_{init}([VO]^{2+} + \text{biotin} \subset \text{Sav})/v_{init}([VO]^{2+}) = 1.5\}$. This observation further supports the hypothesis that the oxidation indeed occurs within the biotin binding pocket. However, due to the poor solubility of the substrate, no saturation kinetics could be achieved (see Supporting Information for details). It is noteworthy that upon using a range of amounts of substrate **3** (50, 100, or 150 equiv of 4-methoxythioanisole versus vanadium), the reaction proceeded with nearly quantitative conversion in all cases. These results suggest that there is no extensive product inhibition under these conditions, although further kinetic studies with a more soluble substrate are required.

X-band EPR experiments performed with $[VO]^{2+} \subset$ WT Sav at pH 2.2 yielded a spectrum very similar to that of $[VO(H_2O)_5]^{2+}$ (Figure 1). Frozen solutions of $[VO(H_2O)_5]^{2+}$-containing complexes display powder pattern electron paramagnetic resonance (EPR) spectra dominated by the hyperfine coupling of the vanadium ion ($S = {}^1/_2$; $I = {}^7/_2$). The spin Hamiltonian parameters used to simulate the experimental data are analogous to those found previously for $[VO(H_2O)_5]^{2+}$,[48] suggesting that the vanadium interacts only via second coordination sphere contacts with streptavidin. In nature, similar examples are provided by molybdate or tungstate anion-binding proteins, in which oriented hydrogen bonds provided by the protein play a major role in conferring exquisite specificity of the proteins toward MO_4^{2-} (M = Mo, W).[49,50] Interestingly, the intensity of the EPR spectrum in the presence of streptavidin is noticeably higher than in its absence, suggesting a shielding of the vanadium complex within the binding site.

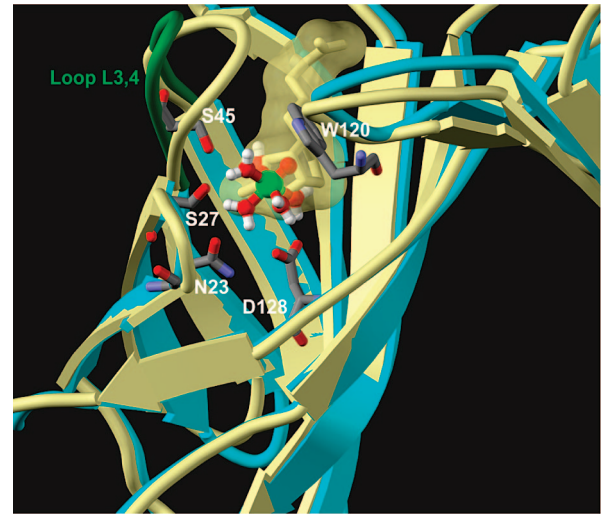

Figure 2. Superimposition of the docked structure $[VO(H_2O)_5]^{2+}$ (ball-and-stick representation) $\subset$ WT Sav (monomers A and D, light blue schematic secondary structure) with the structure of biotin $\subset$ WT Sav at pH = 2.0 (PDB code 2IZG; biotin, yellow stick and yellow transparent surface; monomers A and D, yellow schematic secondary structure). Close-lying D128, N23, S27, and S45 residues are highlighted, as is residue W120 from monomer D, which closes the biotin-binding site of subunit A, as well as the flexible L3,4-loop (green), which was replaced by the corresponding avidin L3,4-loop.

In order to gain an insight on the localization of $[VO(H_2O)_5]^{2+}$ within streptavidin, docking experiments were carried out with a rigid monomeric Sav (PDB code 2IZG;[51] biotin $\subset$ streptavidin at pH = 2.0, the biotin was removed prior to docking; see Supporting Information for computational details). The resulting docked structure $[VO(H_2O)_5]^{2+} \subset$ Sav, with the vanadium moiety located within the biotin-binding pocket, was subsequently allowed to relax in a molecular dynamics simulation on a tetrameric Sav model. The optimized structure is depicted in Figure 2. As can be appreciated, H-donor residues critical for biotin binding (e.g., D128, N23, S27, and S45) are also involved in interactions with $[VO(H_2O)_5]^{2+}$.

It is widely recognized that Asp128 is the most critical residue for the biotin−streptavidin affinity.[4,5,7,52] In the docked structure, Asp128 displays the closest contact to the vanadyl moiety. On

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the basis of this model, we produced and tested the D128A Sav mutant for the sulfoxidation of substrate **3**. This experiment yielded racemic product (Table 1, entry 16), thus strongly supporting the hypothesis that the active vanadyl moiety is located within the biotin-binding pocket during catalysis. Moreover, substituting streptavidin by avidin or bovine serum albumin affords sulfoxidation products with very modest enantioselectivities (Table 1, entries 17 and 18). This suggests that Sav provides a specific binding site for the pentahydrated vanadyl ion.

Inspection of the docked structure revealed that the close-lying L3,4-loop undergoes a significant structural reorganization upon either biotin (the loop is disordered in biotin-free streptavidin) or vanadyl binding (residues 48−52, Figure 2). We produced a chimeric Sav bearing the L3,4-loop of avidin (residues 38−45), abbreviated Aviloop, that retained strong biotin-binding affinity, as previously reported.[53] Despite the lack of selectivity of avidin as host protein, the chimeric artificial metalloenzyme $[VO]^{2+} \subset$ Aviloop afforded improved selectivities compared to WT Sav, both for aliphatic and for aromatic substrates (Table 1, entries 19 and 20). This finding suggests that catalysis indeed takes place in the biotin-binding pocket. Furthermore, it demonstrates that the second-coordination sphere, provided by close-lying residues, influences enantioselectivity.

Conclusion

Incorporation of a vanadyl ion into the biotin-binding pocket of streptavidin affords an artificial enzyme for the enantioselective oxidation of prochiral sulfides. Evidence provided by EPR spectroscopy, docking simulation, and chemical (i.e., addition of biotin) or genetic modification of the host protein suggest that the active precatalyst $[VO(H_2O)_5]^{2+}$ interacts only via second coordination sphere contacts with the biotin-binding pocket of streptavidin. This study demonstrates that specific metal binding can transform a nonenzymatic protein into an enantioselective biocatalyst with synthetic utility. This may thus be regarded as an example of functional promiscuity.[54,55] The findings provide insight into how selective enzymes may first have evolved in nature from noncatalytic scaffolds.[56] Current efforts aim at elucidating the structure and the mechanism of this artificial peroxidase, improving its performance by use of evolution protocols, and investigating the crucial aspect of substrate binding, as well as screening other metals and reactions.

Experimental Section

WT and D128A Sav were produced, purified, and quantified according to ref 57. The chimeric Aviloop DNA was constructed via the Phusion site-directed mutagenesis protocol (Finnzymes, Espoo, Finland) and the protein produced according to ref 57, starting from the mature Sav gene rather than the core Sav gene as reported by Livnah and co-workers.[53] All the substrates were commercially available. Racemic sulfoxides were prepared from the corresponding starting sulfides by oxidation with vanadium sulfate and *t*-BuOOH in CH_2Cl_2 and used for reference purposes.

Typical Procedure for the Asymmetric Sulfoxidation Catalyzed by Vanadium-Loaded Streptavidin. Streptavidin was dissolved in buffer (HCl/KCl 0.05 M, pH 2.2). The protein solution (100 μM final tetrameric concentration) was mixed in a test tube (1 mL capacity) with the vanadium source (200 μM final concentration). After 10 min of incubation time, the substrate was added (1 M stock solution in EtOH, 0.01 M final concentration). The reaction was started by adding the oxidant (3.64 M stock solution in water/EtOH 1/1, 0.05 M final concentration). After 4 h of stirring at room temperature, the reaction mixture was extracted four times with Et_2O. The organic phase was dried over Na_2SO_4 and subjected to HPLC analysis on a Chiralcel OB-H or OD-H column (Daicel Chemical Industries, Tokyo). Analytical data for the sulfoxides are presented in the Supporting Information. The absolute configurations of the sulfoxides were assigned by comparison with ref 47.

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Supporting Information Available: Kinetic measurements, analytical data of the sulfoxides, and computational details. This material is available free of charge via the Internet at http://pubs.acs.org.

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