Breaking Symmetry: Engineering Single-Chain Dimeric Streptavidin as Host for Artificial Metalloenzymes

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ABSTRACT: The biotin–streptavidin technology has been extensively exploited to engineer artificial metalloenzymes (ArMs) that catalyze a dozen different reactions. Despite its versatility, the homotetrameric nature of streptavidin (Sav) and the noncooperative binding of biotinylated cofactors impose two limitations on the genetic optimization of ArMs: (i) point mutations are reflected in all four subunits of Sav, and (ii) the noncooperative binding of biotinylated cofactors to Sav may lead to an erosion in the catalytic performance, depending on the cofactor:biotin-binding site ratio. To address these challenges, we report on our efforts to engineer a (monovalent) single-chain dimeric streptavidin (scdSav) as scaffold for Sav-based ArMs. The versatility of scdSav as host protein is highlighted for the asymmetric transfer hydrogenation of prochiral imines using [{\cpcp}Ir(biot-p-L)-Cl] as cofactor. By capitalizing on a more precise genetic fine-tuning of the biotin-binding vestibule, unrivaled levels of activity and selectivity were achieved for the reduction of challenging prochiral imines. Comparison of the saturation kinetic data and X-ray structures of [{\cpcp}Ir(biot-p-L)-Cl]-scdSav with a structurally related [{\cpcp}Ir(biot-p-L)-Cl]-monovalent scdSav highlights the advantages of the presence of a single biotinylated cofactor precisely localized within the biotin-binding vestibule of the monovalent scdSav. The practicality of scdSav-based ArMs was illustrated for the reduction of the salsolidine precursor (500 mM) to afford (R)-salsolidine in 90% ee and >17 000 TONs. Monovalent scdSav thus provides a versatile scaffold to evolve more efficient ArMs for in vivo catalysis and large-scale applications.

INTRODUCTION

Artificial metalloenzymes (ArMs) are hybrid catalysts combining attractive features of organometallic catalysts (e.g., broad range of catalyzed reactions) and enzymes (e.g., genetically evolvable and compatible with a cellular environment). They are often created by incorporation of a synthetic metallocofactor within a protein scaffold. Thanks to the progress in biotechnology, several scaffolds have proven versatile for the assembly and optimization of ArMs. These include, among others, hemeproteins, transcriptional repressor LmrR,20 carbonic anhydrase,21,22 and streptavidin (Sav).23,24 Due to the high affinity of biotin for (strept)avidin, Sav has proven to be an ideal host for the noncooperative binding of biotinylated cofactors to the two symmetry-related S112 and K121 residues. Accordingly, any mutation of these residues is expected in both Sav monomers (designated SavA and SavB hereafter), thus challenging a precise genetic fine-tuning of the ArM’s performance: it is hard to decipher which of the two symmetry-related mutations (i.e., SavA or SavB) affects (most) the catalytic performance.

An additional challenge concerns the catalytic performance as a function of cofactor occupancy within Sav. Indeed, some ArMs display markedly different catalytic performances as a function of cofactor occupancy within Sav. Indeed, some ArMs display markedly different catalytic performances as a function of cofactor occupancy within Sav.
function of cofactor:Sav ratio. Upon varying the [Cp*Ir(biot-p-L)Cl]:Sav S112A ratio from one to four, the enantioselectivity of the asymmetric transfer hydrogenase toward the salsolidine precursor 1a erodes from 93% ee (R)-2a to 45% ee (R)-2a, Figure 1c,d. In stark contrast, for [Cp*Ir(biot-p-L)Cl]:Sav S112K, the enantioselectivity remains by-and-large constant, irrespective of the above ratio (e.g., 70% ee (S)-2a vs 78% ee (S)-2a upon varying the ratio from one to four). The complications are exacerbated by the noncooperative binding of biotinylated cofactors to Sav: adding 2 equiv of biotinylated cofactor vs homotetrameric Sav affords a Poisson distribution of cofactor occupancy. With in vivo catalysis in mind, whereby the [Sav] is unknown and highly variable, it is desirable to achieve a precise control of ArM’s performance, irrespective of the Sav:cofactor ratio. With these goals in mind, we present herein our efforts toward the engineering of a single chain dimeric streptavidin (scdSav hereafter), enabling the independent mutagenesis of the two neighboring Sav subunits.

### RESULTS AND DISCUSSION

#### Design of scdSav

Circular permutation strategies have been applied to fuse two subunits of avidin while maintaining high biotin-binding affinity. Circularly permuted Sav has also been reported but with a significantly reduced affinity to biotin. Initially, we evaluated a similar approach to engineer circularly permutated scdSav. However, these constructs expressed poorly in E. coli (<1 mg L−1 in culture medium),
hampering thorough characterization and practical implementation in biotechnology. We thus set out to fuse the C-terminus of SavA with the N-terminus of SavB, Figure 1e. Inspection of the X-ray structure of mature Sav (PDB code 2BC3) reveals that the C- and N-termini are remote from each other in the quaternary structure. To connect two Sav subunits, a 26 amino acid linker was thus introduced. In addition, the following points were taken into consideration in the design of scdSav: (i) the DNA sequence homology of the two linked-Sav subunits was minimized to facilitate mutagenesis of each Sav subunit independently; (ii) as the H127 residue forms a π-stacking interaction with its neighboring H127 residue, it was mutated to H127C in the SavB to favor the formation of a disulfide bond with an adjacent scdSav. This ensures the precise assembly of two scdSavs into a single quaternary structure (i.e., (scdSav)2) bearing one disulfide bond and one π−π stacking interaction between the two remaining histidines H127 present in the (scdSav)2 quaternary structure, Figure 1e.43,46 With these considerations in mind, scdSav was engineered using a 26 aa linker between SavA and SavB, with the H127C mutation in the SavB.

To evaluate the suitability of the scdSav design in the context of ArMs, we selected the asymmetric transfer hydrogenation of prochiral imines using [Cp*Ir(biot-p-L)Cl]·scdSav as a test bed (ATHase hereafter). In this context, the nature of the amino acid at position 112 and 121 was repeatedly shown to significantly affect the ATHase performance, both in terms of activity and selectivity.31,33,47,48 In contrast to other ArMs based on the biotin−streptavidin technology,9,23,24 the catalytic performance of the evolved ATHases was shown to critically depend on the cofactor:biotin-binding site ratio, Figure 1c,d. Building on this knowledge, we engineered 33 scdSavs with mutations at both of the S112 and K121 positions. The two S112 positions were independently mutated to either alanine, lysine or arginine. The two K121 were independently mutated to an alanine residue or kept as a lysine. To simplify the labeling of these variants, a 4-letter code is used hereafter: the first 2 letters code for residues 112 and 121 within the SavA and the last 2 letters code for residues 112 and 121 in the SavB, respectively. Accordingly, scdSav(SKSK) represents the “wild type” scdSav, which includes the 26 aa linker and the H127C mutation in the SavB.

The scdSav(SASK) gene was synthesized and introduced on the pRSFduet-1 plasmid. The other 32 scdSavs were generated by site-directed mutagenesis using the above plasmid. All the 33 scdSavs were overexpressed in E. coli BL21(DE3) using an autoinduction medium and purified by affinity chromatography on an iminobiotin-sepharose matrix (see SI Figure S1 for SDS-PAGE analysis, Table S8 for MS data). For all 33 scdSav variants, 10−50 mg of purified protein was obtained from 1 L
of culture medium. SDS-PAGE analysis of the purified scdSavs revealed the formation of (scdSav)$_2$ (= tetramer of Sav) as the major component, with higher oligomers of scdSav as the minor byproducts (Figure S1). Importantly, all scdSavs maintained their “four equivalents” binding capacity toward biotin-4-fluorescein (B4F).

ATHase Performance of ArMs Based on scdSav. Five prochiral imines 1a–e were selected to evaluate the catalytic performance of the engineered [Cp$^*$Ir(biot-p-L)-Cl]-scdSav. Three isoquinoline derivatives (1a–1c), a 2H-pyrrrole derivative (1d), and quinaldine (1e) were selected as the substrates for the reduction, Scheme 1. With the exception of isoquinoline 1a, these substrates have proven challenging to reduce enantioselectively with ATHases. The results of the ATHase screening for substrates 1a–e are summarized in Scheme 1 (see also Table S1). While the free cofactor [Cp$^*$Ir(biot-p-L)-Cl] affords the corresponding racemic amines 2a–e in low conversion (1–18%), incorporation within WT Sav leads to low to moderate conversion (9–68%) accompanied by modest ee values (30–79%). Upon substituting WT Sav by scdSav(SKSK), the resulting ATHase activity by-and-large remains the same, both in terms of conversion (14–70%) and enantioselectivity (42–79%). Mutagenesis at positions 112 and 121 in both Sav$^a$ and Sav$^b$ markedly altered the catalytic performance. To identify important trends, the results were grouped and analyzed according to the amino acids at each specific position (Figure S2). For residue 112 in Sav$^a$, Ser and Ala clearly outperformed Lys and Arg in terms of conversion for all substrates (Figure S2a). The 112S/A in Sav$^b$ is also more (R)-enantioselective for isoquinoline derivatives (1a–1c) and (S)-enantioselective for 1e (Figure S2b). For residue 121 in Sav$^a$, Ala generally improves the activity while largely maintaining the enantioselectivity for 1a–1c and 1e (Figure S2c,d). The general effects of 112S/A and 121A in Sav$^a$ suggest that they are contributing to the binding of [Cp$^*$Ir(biot-p-L)-Cl] in a more productive conformation, in line with a previous computational study of ATHase based on Sav.35 On the other hand, no general trends could be deduced for residues 112 and 121 in Sav$^b$ (Figure S2e–h). The substrate- and context-specific effects of 112 and 121 in Sav$^b$ suggest that they may contribute to interactions with the substrate and thus fine-tune the activity and enantioselectivity. The best ATHases for each substrate are discussed below. [Cp$^*$Ir(biot-p-L)-Cl]-scdSav(SARK) reduced imine 1a to amine (R)-2a in 100% conversion (400 TON) and 96% ee, and imine 1b to amine (R)-2b in 99% conversion (398 TON) and 93% ee, respectively. In comparison with the ATHases based on homotetrameric Sav, scdSav(SARK) is similar to the benchmark Sav S112A for (R)-2a (full conversion and 96% ee at 5 °C).31 For amine (R)-2b, scdSav(SARK) is markedly more enantioselective than the benchmark SavS112T (full conversion and 59% ee).49 Clearly, the fine-tuned scdSav(SARK) bearing 112S and 121A in Sav$^a$ and 112R and 121K in Sav$^b$ outperforms all the homotetrameric Savs tested to date. For the reduction of the bulky substrate 1c, none of the scdSavs exhibited high stereoselectivity (i.e., ee < 70%). For the reduction of imine 1d, [Cp$^*$Ir(biot-p-L)-Cl]-scdSav(SKAA) produced amine (R)-2d in 90% conversion (360 TON) and 98% ee, outperforming the best ATHase with homotetrameric Sav (68% conversion and 77% ee with WT Sav). Such high enantioselectivity (i.e., ee ≥ 95%) is challenging to achieve with ArMs. Again, scdSav(SKAA) bearing 112S and 121K in Sav$^a$ and 112A and 121A in Sav$^b$ outperforms all homotetrameric Savs tested to date. Quinaldine (1e) was only moderately reduced (conversion < 50%) by the ATHases under the experimental conditions. Nevertheless, chiral amine (S)-2e was produced in 91% ee with [Cp$^*$Ir(biot-p-L)-Cl]-scdSav(SARK). In recent years, a number of imine reductase enzymes (IREDs) have been discovered and applied to reduce many cyclic imines to chiral amines with high enantioselectivity.50–54 More recently, several IREDs have been found to catalyze reductive amination,55 i.e., first formation of imines and then reduction to amines, a breakthrough in the enzymatic generation of amines. ATHases have widely been used as a testbed for the development of ArMs.12,31,55,41,48,49 In comparison to evolved IREDs recently reported, ATHases typically display more modest catalytic performances.

Engineering a Monovalent scdSav. In light of the high activity and enantioselectivity for the imine reduction, we selected the double mutant scdSav(SARK) to engineer a monovalent scdSav to investigate the influence of a neighboring cofactor on the catalytic performance of ATHases based on scdSav. Binding of a second biotinylated cofactor in the neighboring Sav subunit often leads to substantial erosion in rate and enantioselectivity.55 To determine the biotin-binding stoichiometry of scdSav, a biotin-4-fluorescein (B4F hereafter) titration was performed, relying on the intersection of the two linear segments of the titration curve to determine the binding stoichiometry.56 scdSav(SASK) and scdSav(SARK) bind 1.9 ± 0.1 and 2.0 ± 0.1 B4F per scdSav respectively (Figure S3a,b). To generate a monovalent scdSav,
The ee values were determined after 48 h with [1a] = 25 mM. The $k_{cat}$ values are normalized to the concentration of [Cp*Ir(biot-p-L)-Cl].  Not available. Error margins represent ± standard deviation resulting from two independent experiments.

while maintaining the overall topology within the biotin-binding vestibule, two reported sets of mutations were evaluated to knockout the biotin-binding capacity in Sav\textsuperscript{8}. The following mutants were produced: scdSav(SARK) with additional N23A/S27D in Sav\textsuperscript{8} (scdSav(SARK)mv1 hereafter) and scdSav(SARK) with additional N23A/S27D/D128A in Sav\textsuperscript{8} (scdSav(SARK)mv2 hereafter). The N23A/S27D and the D128A mutants have been reported to disrupt key H-bonding interactions between Sav and the ureido oxygen and ureido nitrogen by Cantor\textsuperscript{57} and Salemme,\textsuperscript{58} respectively. Gratifyingly, BF4 titration of the resulting scdSav(SARK)mv1 and scdSav(SARK)mv2 revealed the monovalent character of these constructs: 1.1 ± 0.1 and 1.2 ± 0.1 biotin-binding sites were determined for scdSav(SARK)mv1 and scdSav(SARK)mv2, respectively (Figure S3c,d).

Saturation Kinetics of ATHases Based on scdSav. To gain further insight into the catalytic efficiency of ATHases based on scdSav, we determined the saturation kinetics and enantioselectivity for the reduction of imine 1a with ATHases based on scdSav\textsuperscript{4}.

| protein               | [Cp*Ir(biot-p-L)-Cl] (μM) | (R)-2a ee (%)\textsuperscript{b} | $k_{cat}$ (min\textsuperscript{-1})\textsuperscript{c} | $K_m$ (mM) | $K_i$ (mM) |
|-----------------------|--------------------------|----------------------------------|---------------------------------|-------------|-------------|
| no protein            | 50                       | 1                                | 1.3 ± 0.1                       | 57 ± 20     | n.a.\textsuperscript{d} |
| scdSav(SARK)          | 25                       | 94                               | 10.0 ± 0.8                      | 20 ± 5      | 687 ± 189   |
| scdSav(SARK)          | 50                       | 95                               | 12.5 ± 1.2                      | 32 ± 7      | 359 ± 79    |
| scdSav(SARK)          | 75                       | 87                               | 4.6 ± 0.6                       | 23 ± 8      | 474 ± 158   |
| scdSav(SARK)          | 100                      | 74                               | 2.2 ± 0.3                       | 38 ± 13     | 614 ± 285   |
| scdSav(SARK)mv1       | 25                       | 95                               | 16.0 ± 1.7                      | 31 ± 7      | 333 ± 77    |
| scdSav(SARK)mv1       | 50                       | 96                               | 19.8 ± 2.9                      | 36 ± 11     | 294 ± 89    |
| scdSav(SARK)mv2       | 25                       | 94                               | 23.6 ± 3.0                      | 52 ± 12     | 298 ± 74    |
| scdSav(SARK)mv2       | 50                       | 95                               | 21.0 ± 2.7                      | 42 ± 11     | 513 ± 168   |

The reaction conditions are described in Figure 2. The kinetic parameters were obtained using the Michaelis–Menten and Haldane equations for the free cofactor and the ATHases, respectively. The ee values were determined after 48 h with [1a] = 25 mM. The $k_{cat}$ values are normalized to the concentration of [Cp*Ir(biot-p-L)-Cl].  Not available. Error margins represent ± standard deviation resulting from two independent experiments.

While maintaining the overall topology within the biotin-binding vestibule, two reported sets of mutations were evaluated to knock out the biotin-binding capacity in Sav\textsuperscript{8}. The following mutants were produced: scdSav(SARK) with additional N23A/S27D in Sav\textsuperscript{8} (scdSav(SARK)mv1 hereafter) and scdSav(SARK) with additional N23A/S27D/D128A in Sav\textsuperscript{8} (scdSav(SARK)mv2 hereafter). The N23A/S27D and the D128A mutants have been reported to disrupt key H-bonding interactions between Sav and the ureido oxygen and ureido nitrogen by Cantor\textsuperscript{57} and Salemme,\textsuperscript{58} respectively. Gratifyingly, BF4 titration of the resulting scdSav(SARK)mv1 and scdSav(SARK)mv2 revealed the monovalent character of these constructs: 1.1 ± 0.1 and 1.2 ± 0.1 biotin-binding sites were determined for scdSav(SARK)mv1 and scdSav(SARK)mv2, respectively (Figure S3c,d).

Saturation Kinetics of ATHases Based on scdSav. To gain further insight into the catalytic efficiency of ATHases based on scdSav, we determined the saturation kinetics and enantioselectivity for the reduction of imine 1a to chiral amine (R)-2a by [Cp*Ir(biot-p-L)-Cl]-scdSav(SARK) and its two monovalent variants with varying Ir:scdSav ratios. For all measurements, the concentration of scdSav was kept at 50 μM, whereas 25, 50, 75, 100 μM [Cp*Ir(biot-p-L)-Cl] were used for scdSav(SARK) (Ir:scdSav ratio = 0.5, 1.0, 1.5, 2.0 respectively), and 25, 50 μM [Cp*Ir(biot-p-L)-Cl] were used for the monovalent Savs scdSav(SARK)mv1 and scdSav(SARK)mv2 (Ir:scdSav ratio = 0.5, 1.0 respectively). As summarized in Figure 2 and Table 1, all the ATHases gave rise to high enantioselectivities and pronounced rate-accelerations to afford up to 96% ee (R)-2a and up to a 10-fold higher $k_{cat}$ compared to the free cofactor [Cp*Ir(biot-p-L)-Cl]. For scdSav(SARK), capable of binding up to two biotinylated cofactors per scdSav, the reaction rate ($k_{cat}$) markedly decreased and the enantioselectivity was moderately reduced upon increasing the Ir:scdSav beyond one. The erosion of rate and enantioselectivity are in accordance with a previous study of [Cp*Ir(biot-p-L)-Cl]-Sav S112A,\textsuperscript{35} suggesting that the binding of an additional [Cp*Ir(biot-p-L)-Cl] in the neighboring Sav subunit may cause a significant conformational change of the existing cofactor into a catalytically less favorable conformation.\textsuperscript{35} Because scdSav(SARK)mv1 and scdSav(SARK)mv2 are monovalent, the biotinylated cofactor cannot bind to the second Sav subunit. Thus, no detrimental effects were observed upon increasing the cofactor to protein ratio up to full saturation. In addition, when Ir:scdSav ≤ 1, a higher reaction rate was observed for monovalent scdSav ($k_{cat} = 16.0–23.6$ min\textsuperscript{-1}) than that of divalent scdSav ($k_{cat} = 10.0–12.5$ min\textsuperscript{-1}) and previous homotetrameric Sav S112A ($k_{cat} = 11.4–14.1$ min\textsuperscript{-1}).\textsuperscript{35} The superiority of monovalent scdSav may be traced back to the fact that all the bound cofactor resides in a catalytically active conformation for the monovalent scdSav. In contrast, and in light of the non-cooperative binding of the cofactor within Sav, even at Ir:scdSav ≤ 1, a portion of the cofactor [Cp*Ir(biot-p-L)-Cl] binds in an unproductive conformation, leading to an erosion in rate.

Structural Characterization of scdSav ARMs by X-ray Crystallography. To gain structural insight into the ATHases based on the single chain dimeric streptavidin constructs, both scdSav(SARK) and scdSav(SARK)mv2 were crystallized by sitting drop vapor diffusion (2.5 μL 26 mg/mL (scdSav), 10 mM sodium phosphate buffer with 150 mM NaCl, pH 7 was mixed with precipitation buffer 2.5 μL 2 L (NH₄)₂SO₄, 0.1 M Na-Acetate, pH 4). The resulting apo crystals were soaked with excess [Cp*Ir(biot-p-L)-Cl] (dissolved in DMSO). The soaked yellow crystals were cryoprotected with 25% glycerol and flash frozen in liquid nitrogen. The structure was solved by molecular replacement using the PDB structure 3PK2 as a molecular model. Residual electron density in the $P_{1}–P_{2}$ map was observed in the biotin-binding pocket and in the biotin-binding vestibule. Anomalous dispersion density was observed in the biotin-binding vestibule. Modeling of cofactor [Cp*Ir(biot-p-L)-Cl] into the electron density projected the iridium in the position of the anomalous density peak. The X-ray structures reveal, as anticipated, that [Cp*Ir(biot-p-L)-Cl]-scdSav(SARK) and [Cp*Ir(biot-p-L)-Cl]-scdSav(SARK)mv2 differ in their Ir:scdSav ratios. For the biological dimer of scdSav(SARK) (i.e., (scdSav(SARK))₂), both biotin-binding sites present in the single chain dimer are occupied (i.e., amounting to four cofactors for the functional dimeric assembly (scdSav)₂), Figure 3a. In stark contrast, the biological dimer of monovalent scdSav(SARK)mv2 (i.e., (scdSav(SARK)mv2)₂) contains only one [Cp*Ir(biot-p-L)-Cl] cofactor per single chain dimer (i.e., two cofactors for the functional dimeric assembly of (scdSav)₂), Figure 3b (see Figure S18 for the anomalous electron density of the iridium atoms). To accommodate the two cofactors within the biotin-binding vestibule of scdSav(SARK), the neighboring cofactors are forced to adopt two different conformations C1 and CII, each with a 50% occupancy and both in the (S₅)₅− configuration with a Cl⁻ coordinated to Ir. Severe steric clashes prevent neighboring cofactors from occupying two C1 conformations.
simultaneously, Figure 3a. For \([\text{Cp}^*\text{Ir(biot-p-L)Cl}] \cdot \text{scdSav(SARK)}\) in complex with \([\text{Cp}^*\text{Ir(biot-p-L)Cl}] \cdot \text{scdSav(SARK)}\) and \([\text{Cp}^*\text{Ir(biot-p-L)Cl}] \cdot \text{scdSav(SARK)}\) with bound \([\text{Cp}^*\text{Ir(biot-p-L)Cl}] \cdot \text{scdSav(SARK)}\) cofactor. The \text{scdSav} proteins are displayed as surface representation and colored by protein chains. The cofactors are represented as stick models and the Ir-atom as orange sphere; nitrogen, blue; oxygen, red; sulfur, yellow. See Figure S21 for stereo view. (a) The two cofactor conformations (CI and CII) of \([\text{Cp}^*\text{Ir(biot-p-L)Cl}] \) bound to each binding site are depicted. Both conformations CI and CII are occupied at 50%. Selected distances between the two cofactors are highlighted in yellow dashes. (b) For monovalent \text{scdSav(SARK)} only one binding site per \(\text{scdSav})\) is occupied by \([\text{Cp}^*\text{Ir(biot-p-L)Cl}] \). (c) The electron density map \(2F_o - F_c\) of the residues stabilizing the interactions of a homodimeric \(\text{scdSav(SARK)}\) (c) the disulfide bond formed by the two C311-residues; (d) the \(\pi-\pi\) stacking interaction of the two H127-residues. The protein is displayed in cartoon mode and colored by protein chain. The interacting amino acids are shown as sticks: nitrogen, blue; sulfur, yellow. (e) Schematic illustration of the (nearly) statistical orientation of crystal packing of \([\text{Cp}^*\text{Ir(biot-p-L)Cl}] \cdot \text{scdSav(SARK)}\) reflected in the apparent occupation of both biotin-binding sites, albeit with partial occupancy. The orientation \(\text{scdSav(SARK)}\) highlighted in blue in the crystal packing leads to an apparent partial occupation of all four biotin-binding sites, although only the Sav^A site is effectively occupied.

As can be appreciated in Figure 3a, the protein environment surrounding the two cofactor conformations CI and CII for \text{scdSav(SARK)} is markedly different (see also Figure S21 for the stereo view of the environment). As predicted by Maréchal and co-workers, the solvent-exposed CII conformation leads to a decrease in affinity and enantioselectivity, see Figure 2.33,59 As the catalytically less efficient conformation CII is not observed in \([\text{Cp}^*\text{Ir(biot-p-L)Cl}] \cdot \text{scdSav(SARK)}\) with bound \([\text{Cp}^*\text{Ir(biot-p-L)Cl}] \cdot \text{scdSav(SARK)}\) compared to Sav S112K. This suggests that CII resides in a shallower potential energy well, provided by the protein, than conformation CI.

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Table 2. Optimization of the Reduction of Dihydroisoquinoline 1a with ATHases Based on scdSav+*  

| protein       | [Cp*Ir(biot-p-L)Cl] (μM) | 1a conc. (mM) | temp. (°C) | conv. (%) | (R)-2a ee (%) | TON |
|---------------|-------------------------|--------------|------------|-----------|---------------|-----|
| scdSav(SARK)  | 5                       | 10           | 25         | 95        | 96            | 1910 |
| scdSav(SARK)  | 5                       | 50           | 25         | 73        | 92            | 7320 |
| scdSav(SARK)  | 5                       | 100          | 25         | 53        | 90            | 10700|
| scdSav(SARK)mv1 | 5                     | 100          | 50         | 78        | 86            | 15600|
| scdSav(SARK)mv2 | 5                 | 100          | 50         | 84        | 89            | 16900|
| scdSav(SARK)  | 50                      | 500          | 50         | 88        | 90            | 17700|
| scdSav(SARK)mv1 | 50                 | 500          | 50         | 95        | 87            | 9540 |
| scdSav(SARK)mv2 | 50               | 500          | 50         | 95        | 87            | 9510 |

*Reaction conditions: 10–500 mM substrate, 5–50 μM [Cp*Ir(biot-p-L)Cl], 5–50 μM scdSav (= concentration of [Cp*Ir(biot-p-L)Cl]), 0.6 M MOPS, 3 M formate, pH 7.0, 25–50 °C, 48 h. b 1a (100 mM aliquots) was fed for five consecutive times at t = 0, 2, 5, 10, and 20 h.

Ir (i.e., S₀ with a Cl− coordinated to Ir), these preferentially lead to the same enantiomer-enriched amine, albeit with different levels of enantioselectivity.

Although the DNA sequence homology of the fused subunits was minimized to simplify mutagenesis efforts, the amino acid sequence of both subunits is nearly identical: the fold, and the corresponding electron density observed for each subunit of the scdSav are very similar. Accordingly, in the crystal packing, each tetramer can occupy two orientations leading to a mixed electron density. Nonetheless, we observed the crystal packing, each tetramer can occupy two orientations for each subunit of the scdSav are very similar. We thus assigned the electron density of N23A/S27D/H127C/D128A and the (SARK)mv2 in the crystals resulting in diastereotopic electron densities. In the homodimeric structure is depicted in Figure 3c. The 26-amino acid linker distribution of the tetramer orientation and the resulting mixed references for the electron density observed for these amino acids.

Reaction Optimization and Preparative Scale Synthesis. Having identified the best (scdSav)_2 scaffolds for the reduction of imines, we sought to optimize the conditions for the ATHases. For this purpose, the effect of temperature, pH, and substrate concentration were evaluated. For the asymmetric reduction of imine 1a, pH = 7.0 proved best both in terms of conversion and enantioselectivity (Figure S5). Upon increasing the temperature from 25 to 50 °C, higher turnover frequencies (TOF) and TONs were observed, at the cost of a slightly eroded ee (Table 2 and Figure S6). Next, the catalytic potential of [Cp*Ir(biot-p-L)Cl]·scdSav(SARK) and both its monovalent isoforms were tested (Table 2). At 50 °C and with [Cp*Ir(biot-p-L)Cl] (5 μM), scdSav(SARK) (5 μM) and substrate 1a (100 mM), amine (R)-2a is produced in 78% conversion (TON = 15 600) and 86% ee. With [Cp*Ir(biot-p-L)Cl]·scdSav(SARK)mv2, the conversion and the enantioselectivity increases to 88% (TON = 17 700) and 90% ee (R)-2a. This TON is significantly higher than that of [Cp*Ir(biot-p-L)Cl]·Savr S112A (TON = 4000). High substrate loading is often challenging for ATHases. Indeed, for the [Cp*Ir(biot-p-L)Cl]·scdSav(SARK) isoforms, we determined substrate inhibition (Kᵢ = 294–687 mM, Table 1). To circumvent this challenge, we applied a slow feed of substrate 1a. To our delight, substrate 1a (500 mM) was reduced to amine (R)-2a in 95% conversion and 87% ee with [Cp*Ir(biot-p-L)Cl]·scdSav(SARK)mv2 (50 μM) (Table 2, and Figure S7 for the time course). This corresponds to a product concentration of 98.5 g/L, which is similar to the current record of IRED (103.5 g/L) for the same type of reaction.55 A preparative-scale experiment (reaction volume 10 mL) was carried out with substrate 1a (200 mM) and [Cp*Ir(biot-p-L)Cl]·scdSav(SARK)mv1 (50 μM) in a conical flask: 408.2 mg of amine (R)-2a was isolated in 91% ee and 98.5% yield (see Figure S8 for the time course). The same preparative reaction was also performed with [Cp*Ir(biot-p-L)Cl]·scdSav(SARK)mv2 (50 μM) and 399.3 mg of amine (R)-2a was isolated in 92% ee and 96.3% yield (Figure S8). Importantly, essentially pure (R)-salsolidine 2a could be obtained following a simple extraction procedure (>95% purity by 1H NMR, see Figure S16 and S17).

The asymmetric reduction of other prochiral imines 1b, 1d, and 1e was also investigated, Scheme 2. By evaluating different loading of 1b and reaction temperatures (Table S2), we found that imine 1b (100 mM) was quantitatively reduced to amine (R)-2b in 91% ee with [Cp*Ir(biot-p-L)Cl]·scdSav(SARK)-
mv2 (50 μM), corresponding to a TON of 2000. Similarly, imine 1d (100 mM) was reduced to amine (R)-2d in 99% conversion and 96% ee with [Cp*Ir(biot-p-L)-Cl]−scdSav(SKAA) (50 μM), corresponding to a TON of 1980 (Table S3). Quinaldine (1e) proved challenging to reduce: only 24% conversion to (S)-2e was obtained with [Cp*Ir(biot-p-L)-Cl]−scdSav(SARK) (Scheme 1). Upon increasing the reaction temperature to 50 °C and relying on scdSav(SARK)mv2 (Table S4), 98% conversion (TON = 195) and 91% ee (S)-2e could be achieved with 50 μM [Cp*Ir(biot-p-L)-Cl]−scdSav(SARK)mv2.

### CONCLUSION AND OUTLOOK

With the aims of expanding the genetic optimization potential as well as resolving the issue related to multiple cofactor binding within the biotin-binding vestibule in ArMs based on the biotin–streptavidin technology, we engineered a single-chain dimeric streptavidin. As both SavA 112 and SavB 112 residues as well as SavA 121 and SavB 121 lie in the immediate proximity of the biotinylated metal moiety (Figure 1), the possibility of varying these four critical residues individually (to potentially generate 204 = 160 000 scdSav mutants) significantly expands the genetic diversity of ArMs based on scdSav.50−62 The potential of this strategy was highlighted by evaluating the ATHase activity of 33 different scdSavs toward challenging substrates 1a–e. Gratifyingly, the resulting ATHases outperformed homotetrameric Sav-based ATHases, both in terms of activity (e.g., TON) and selectivity. Further improvements were observed in the presence of monovalent scdSav(SARK)mv1 and scdSav(SARK)mv2. Enzyme kinetics revealed that the monovalent scdSav outperformed the related divalent scdSav for the reduction of substrates 1a, 1b, and 1e, as the interference of neighboring cofactors is lifted, as highlighted in the X-ray structure of [Cp*Ir(biot-p-L)-Cl]−scdSav(SASK) and [Cp*Ir(biot-p-L)-Cl]−scdSav(SARK)mv2. The practicality of [Cp*Ir(biot-p-L)-Cl]−scdSav(SARK)mv2 was illustrated for the reduction of imine [1a] = 500 mM to afford salolsidine (R)-2a in 90% ee and >17 000 TONs. In view of the high recombinant Sav production yields obtained in fed-batch mode (e.g., > 8 g/L soluble homotetrameric Sav),37 we hope that these findings will contribute to large scale applications of this technology. With in vivo catalysis in mind,39–41,63–65 the use of a monovalent scdSav may prove versatile as the catalytic performance of the ArMs is independent of cofactor:Sav ratios. This may also prove useful in other advanced biotechnological applications.66

### ASSOCIATED CONTENT

- **Supporting Information**
  The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.9b06923.

Experimental procedures, crystallographic data, spectroscopic data, supporting figures and tables (PDF)

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**Notes**

The authors declare no competing financial interest.

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