Improving the enantioselectivity of artificial transfer hydrogenases based on the biotin–streptavidin technology by combinations of point mutations

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ABSTRACT

Artificial metalloenzymes based on the incorporation of biotinylated ruthenium piano–stool complexes within streptavidin can be readily optimized by chemical or genetic means. We performed genetic modifications of such artificial metalloenzymes for the transfer hydrogenation of aromatic ketones, by combining targeted point mutations of the host protein. Upon using the P64G-L124V double mutant of streptavidin in combination with the $[\eta^6$-(p-cymene)Ru(**Biot-p-L**)Cl] complex, the enantioselectivity can be increased up to 98% ee (R) for the reduction of p-methylacetophenone, which is the highest selectivity obtained up to date with an artificial transfer hydrogenase.

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1. Introduction

Since the innovative systems designed by Whitesides and Kaiser about three decades ago [1,2], the creation of artificial metalloenzymes as novel approaches to enantioselective catalysts has received considerable interest [3–8]. In this context, hybrid catalysts based on the biotin–streptavidin technology offer new perspectives for protein engineering by combining chemical and genetic modifications to create new activities and to fine-tune the selectivity [9]. Recently, we have exploited the potential of chemogenetic optimization to create enantioselective transfer hydrogenases for the reduction of aromatic and aliphatic ketones [10–12]. Initial studies have allowed the identification of biotinylated Ru d^6 piano–stool complexes, which, in the presence of streptavidin, catalyzed the aqueous transfer hydrogenation of acetophenone derivatives [10]. In a first round of evolution, based on chemical modifications at the organometallic moiety and on saturation mutagenesis at the close-lying 112 position,

high (R)- and moderate (S)-enantioselectivities were obtained for the reduction of aromatic ketones (up to 97% ee (R)) [11]. On the basis of the crystal structure of such a transfer hydrogenase, additional short contacts between the biotinylated catalyst and amino acid side chains were identified. During a consecutive step of designed evolution, starting from an (R)- and an (S)-selective mutant, saturation mutagenesis at two targeted residues, K121 and L124, afforded selective transfer hydrogenases for the reduction of the challenging aliphatic ketones. Increased (S)-selectivities could also be obtained for some aromatic substrates (up to 92% ee (S)) [12].

Herein, we report on our efforts to improve the enantioselectivities of these enzymes by exploring new site-directed mutations around the active site, as well as by combining the effects of beneficial mutations. We tested the influence of several simple and double streptavidin mutants on the activity and selectivity of the catalytic transfer hydrogenation of p-methylacetophenone **1** and α-tetralone **2**, as these bulky substrates gave the best enantioselectivities during previous experiments [11]. Two organometallic moieties were used in this study: $[\eta^6$-(p-cymene)Ru(**Biot-p-L**)Cl] and $[\eta^6$-(benzene)Ru(**Biot-p-L**)Cl] (Scheme 1), identified previously as mostly (R)-, respectively (S)-selective in combination with the streptavidin isoforms.

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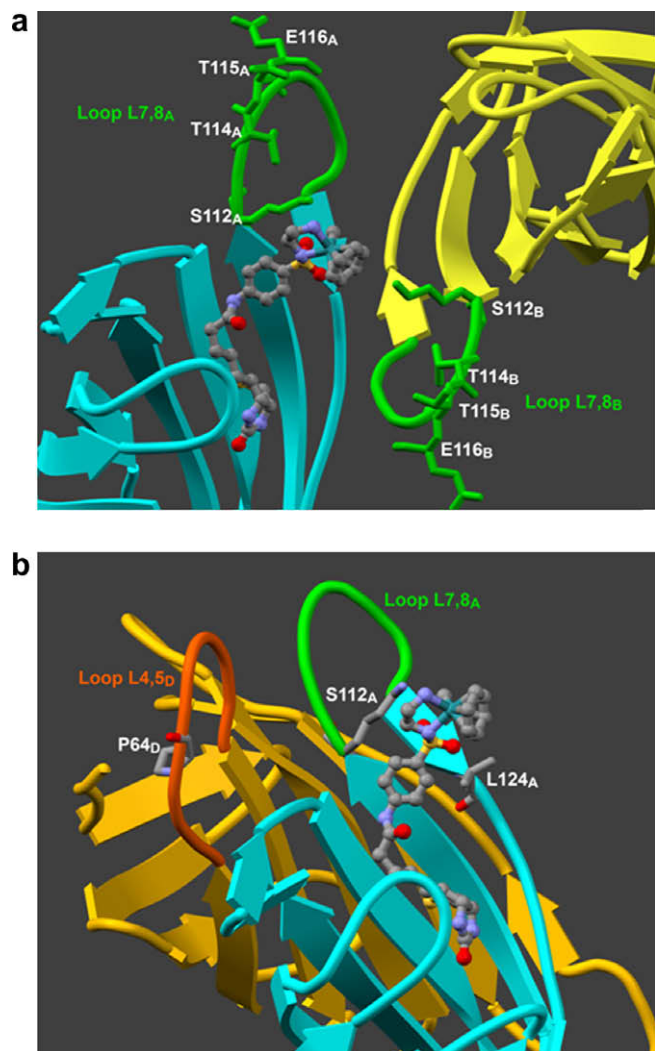E-mail address: thomas.ward@unibas.ch (T.R. Ward).

Fig. 1. Close-up views of the crystal structure of [η^6-(benzene)Ru(**Biot**-***p***-**L**)Cl]⊂S112K Sav tetramer [12]. Only monomer A is occupied by the biotinylated ruthenium complex. (a) The L7,8 loops of monomers A (blue) and B (yellow) are highlighted in green; residues S112, T114, T115 and E116 of both monomers are situated in the proximity of the catalytic site. (b) The neighboring monomers A (blue) and D (orange) are highlighted; the L4,5 loop (red) of monomer D, containing the P64 residue, is situated in the proximity of the L7,8 loop (green) of monomer A; the L124 residue of monomer A is also highlighted. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2. Experimental

2.1. Protein expression and purification

Streptavidin mutants were produced, purified and quantified according to Ref. [13].

2.2. Catalytic runs

The substrates were commercially available. The biotinylated ruthenium complexes, [η^6-(*p*-cymene)Ru(**Biot**-***p***-**L**)Cl] and [η^6-(benzene)Ru(**Biot**-***p***-**L**)Cl], were synthesized as previously published [11]. Lyophilized streptavidin was dissolved in milliQ water (100 μM tetrameric concentration) and the solution was thoroughly degassed (nitrogen flushed during 2 h). The degassed protein (450 μL solution, 0.045 μmol of tetrameric Sav) was mixed in a test tube with the precursor complex [η^6-(arene)Ru(**Biot**-***p***-

L)Cl] (3.8 μL of a 0.0395 M stock solution in DMF, 0.15 μmol Ru) and stirred at room temperature for 10 min. A degassed mixture of HCOONa (1 M) and B(OH)$_3$ (0.85 M) was added to each tube (600 μL mixture, pH adjusted to 6.25), followed by MOPS buffer (200 μL of a 1 M stock solution, pH adjusted to 6.25) and by the corresponding substrate (15 μL of a 1 M stock solution in DMF, 15 μmol). The test tubes were placed in a magnetically stirred multireactor, purged several times with nitrogen and heated at 55 °C for 64 h. After completion, the reaction mixture was extracted with Et$_2$O (4 × 1 mL) and dried over Na$_2$SO$_4$. The organic solution was filtered through a short silicagel plug that was thoroughly washed with Et$_2$O, concentrated and subjected to HPLC analysis.
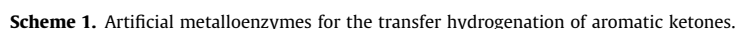
For 1-(*p*-methylphenyl)ethanol, the conversions and ee's were determined using an (*S,S*)-ULMO column (Regis Technologies Inc., IL, USA) with hexane:1,2-dimethoxyethane 100:1.5 at 1 mL/min; t_R = 19.5 min, t_S = 16.6 min (UV detection at 215 nm, absolute configuration assigned by comparison with published results [11]).

For 1,2,3,4-tetrahydro-1-naphthol, the conversions and ee's were determined using an OD-H column (Daicel Chemical Industries, Tokyo) with hexane:isopropanol 100:2 at 0.9 mL/min; t_R = 18.5 min, t_S = 17.2 min (UV detection at 215 nm, absolute configuration assigned by comparison with a commercial enantiopure sample).

3. Results and discussion

The crystal structure of the [η^6-(benzene)Ru(**Biot**-***p***-**L**)Cl]⊂S112K Sav tetramer (the inclusion symbol ⊂ refers to the supramolecular incorporation of the biotinylated ruthenium complex into streptavidin) revealed that the residues of the L7,8 loop (residues 112–121) of both streptavidin monomers A and B were situated in proximity to the catalytic site (Fig. 1a). Therefore, site-directed mutations in this region were expected to influence catalysis by interactions between the protein and the substrate or the ruthenium complex. Previous results allowed the identification of [η^6-(*p*-cymene)Ru(**Biot**-***p***-**L**)Cl]⊂S112A as a highly (*R*)-enantioselective artificial enzyme [11]. In order to perturb the loop structure, three neighboring polar amino acid residues from this loop were mutated with glycine or alanine residues and the new Sav variants, T114G Sav, T115A Sav and E116A Sav, were tested in catalysis in the presence of the two biotinylated Ru complexes. The T114G Sav mutation has a significant influence on the reaction outcome: in combination with [η^6-(*p*-cymene)Ru(**Biot**-***p***-**L**)Cl], this mutation appears as beneficial for enantioselectivity when compared to WT Sav (Table 1, entries 1–4), while the (*S*)-enantioselectivity for the reduction of α-tetralone is increased to 84% ee (*S*) when using [η^6-(benzene)Ru(**Biot**-***p***-**L**)Cl]⊂T114G Sav (Table 1, entries 5–6). The T115A and E116A mutations were not as effective as T114G Sav in terms of enantioselectivity improvement (Table 1, entries 7–8). To further increase the selectivity, we combined the S112A and the T114G mutations, but the resulting double mutant afforded lower conversion and selectivity than the corresponding single mutants (Table 1, entries 9–12). This antagonistic behavior could be due to the fact that the 112 and 114 amino acids lie too close to each other and thus the two modifications might drastically perturb the enantioselective environment around the catalytic site.

Preliminary experiments investigating the effect of genetic modifications of the host protein on the reduction of acetophenone derivatives showed that the streptavidin mutant with the most remote site of mutation (P64G Sav) significantly increased enantioselectivity, while the closest-lying mutation (S112G Sav) afforded a marked decrease in enantioselectivity, although with higher conversions (Table 1, entries 13–14). Interestingly, an enhanced activity and selectivity was observed upon combination

Scheme 1. Artificial metalloenzymes for the transfer hydrogenation of aromatic ketones.

Table 1
Transfer hydrogenation of ketones **1** and **2** catalyzed by different artificial metalloenzymes.

Entry	Sav isoform	Complex	Substrate	Conversion (%)	ee (%)
1	WT	[η^6-(p-Cymene)Ru(**Biot-p-L**)Cl]	**1**	86	91 (R)
2	T114G	[η^6-(p-Cymene)Ru(**Biot-p-L**)Cl]	**1**	85	95 (R)
3	WT	[η^6-(p-Cymene)Ru(**Biot-p-L**)Cl]	**2**	48	86 (R)
4	T114G	[η^6-(p-Cymene)Ru(**Biot-p-L**)Cl]	**2**	42	94 (R)
5	WT	[η^6-(Benzene)Ru(**Biot-p-L**)Cl]	**2**	15	58 (S)
6	T114G	[η^6-(Benzene)Ru(**Biot-p-L**)Cl]	**2**	24	84 (S)
7	T115A	[η^6-(p-Cymene)Ru(**Biot-p-L**)Cl]	**2**	37	86 (R)
8	E116A	[η^6-(p-Cymene)Ru(**Biot-p-L**)Cl]	**2**	20	83 (R)
9	S112A	[η^6-(p-Cymene)Ru(**Biot-p-L**)Cl]	**1**	98	91 (R)
10	S112A-T114G	[η^6-(p-Cymene)Ru(**Biot-p-L**)Cl]	**1**	71	90 (R)
11	S112A	[η^6-(p-Cymene)Ru(**Biot-p-L**)Cl]	**2**	91	91 (R)
12	S112A-T114G	[η^6-(p-Cymene)Ru(**Biot-p-L**)Cl]	**2**	46	86 (R)
13	S112G	[η^6-(p-Cymene)Ru(**Biot-p-L**)Cl]	**2**	45	36 (R)
14	P64G	[η^6-(p-Cymene)Ru(**Biot-p-L**)Cl]	**2**	39	90 (R)
15	S112G-P64G	[η^6-(p-Cymene)Ru(**Biot-p-L**)Cl]	**2**	99	95 (R)
16	P64G	[η^6-(p-Cymene)Ru(**Biot-p-L**)Cl]	**1**	92	94 (R)
17	L124V	[η^6-(p-Cymene)Ru(**Biot-p-L**)Cl]	**1**	99	96 (R)
18	L124V-P64G	[η^6-(p-Cymene)Ru(**Biot-p-L**)Cl]	**1**	98	98 (R)
19	L124V	[η^6-(p-Cymene)Ru(**Biot-p-L**)Cl]	**2**	20	87 (R)
20	L124V-P64G	[η^6-(p-Cymene)Ru(**Biot-p-L**)Cl]	**2**	18	94 (R)

of the two mutations (Table 1, entry 15). As can be appreciated from the crystal structure of the [η^6-(benzene)Ru(**Biot-p-L**)Cl]⊂S112K Sav tetramer (Fig. 1b) P64 amino acid from monomer A containing the organometallic fragment does not lie in the proximity of the catalytic site. In contrast, the L4,5 loop of the neighboring monomer D, containing the P64 residue, seems to have a close contact with the L7,8 loop from monomer A, which is positioned close to the Ru complex (Fig. 1b). Replacing the proline amino acid with a glycine might influence the interactions between the two loops and hence, the enantioselectivity outcome.

In this context, we reasoned that a combination between two individual amino acid alterations with improved enantioselectivity might display an additive effect, if one of the mutations does not drastically perturb the other. Thus, we envisaged combining the P64G mutation with the most efficient Sav isoforms identified during the rounds of designed evolution at positions 112, 121 and 124,

in the attempt to further optimize the performances of the artificial metalloenzymes. The most selective Sav variant for the reduction of aromatic ketones was L124V Sav. The double mutant P64G-L124V Sav afforded high production levels and could be easily purified in milligram quantities. Its evaluation in combination with [η^6-(p-cymene)Ru(**Biot-p-L**)Cl] for the transfer hydrogenation of substrates **1** and **2** afforded the highest level of enantioselectivity obtained with the artificial transfer hydrogenases (98% ee (R) for the reduction of p-methylacetophenone **1**, Table 1, entry 18).

4. Conclusion

In summary, we tested the effect of several genetic modifications on the transfer hydrogenation of the aromatic ketones **1** and **2** using biotinylated ruthenium complexes incorporated into streptavidin. Exploring mutations in loop L7,8, a noticeable

increase in enantioselectivity compared with WT Sav was obtained when using the T114G Sav mutant in combination with [η^6-(p-cymene)Ru(**Biot-p-L**)Cl]. However, this T114G mutation did not provide any additional improvement when combined with S112A, another independently beneficial mutation on the same loop. In contrast, upon combining the beneficial P64G mutation, which is on a more distant loop that is not thought to interact directly with the catalytic moiety, with other beneficial mutations in the region of the active site (L124V or S112G), the enantioselectivity was increased up to 98% ee (R) in the case of the reduction of p-methylacetophenone. These results pave the way towards the generation of larger libraries of streptavidin isoforms with multiple mutations in the vicinity of the ruthenium.

Acknowledgements

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