Turning a riboflavin-binding protein into a self-sufficient monooxygenase by cofactor redesign†

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By cofactor redesign, self-sufficient monooxygenases could be prepared. Tight binding of N-alkylated flavins to riboflavin-binding protein results in the creation of artificial flavoenzymes capable of H₂O₂-driven enantioselective sulfoxidations. By altering the flavin structure, opposite enantioselectivities could be achieved, in accordance with the binding mode predicted by in silico flavin-protein docking of the unnatural flavin cofactors. The study shows that cofactor redesign is a viable approach to create artificial flavoenzymes with unprecedented activities.

Monooxygenases perform selective oxidations of organic substrates under environmentally benign conditions.1 Due to this and their chemo-, regio- and/or enantioselectivity, monooxygenases are regarded as extremely valuable biocatalysts. Nonetheless, the applicability of monooxygenases is often hampered by their dependence on nicotinamide cofactors (NAD(P)H). These cofactors are required to deliver electrons to the oxidizing cofactor, e.g. heme in P450 monooxygenases and flavin in flavoprotein monooxygenases, enabling the enzymes to utilize dioxygen for oxygenations. Several strategies to circumvent the stoichiometric use of nicotinamide cofactors have been reported.2 Of these approaches, the use of additional biocatalysts in combination with a sacrificial cosubstrate has been the most effective.3 Nevertheless, the use of cofactor-independent monooxygenases is far less complicated, as has been demonstrated for a few monooxygenases. Thus, light can be used to reduce cytochrome P450 and flavoprotein monooxygenases.4 Cytochrome P450 monooxygenases have also been shown to operate at the expense of peroxides, thereby bypassing the cofactor-dependent heme reduction and subsequent oxygenation of the heme iron center.5 Whereas these methodologies are viable, they are not very efficient. We here report on a novel concept for the design of artificial self-sufficient flavoprotein monooxygenases. By flavin cofactor redesign several artificial flavoenzymes were created that function as peroxide-driven monooxygenases, capable of enantioselective sulfoxidations.

Flavoprotein monooxygenases represent a major group within the superfamily of monooxygenases.6 These biocatalysts exploit the chemical versatility of the flavin cofactor to perform selective oxygenations. A key enzyme intermediate in the catalytic cycle is the so-called 4α-hydroperoxyflavin (Scheme 1, bottom), formed by reduction of the flavin, typically at the expense of NAD(P)H, and subsequent reaction with dioxygen. While flavoprotein monooxygenases are able to form and stabilize such intermediates,7 other flavoproteins are unable to do so and it is known that peroxylavins quickly decay when free in solution.7 The details on how enzymes are able to tune the stabilisation of these specific enzyme intermediates still remain obscure.8

Analogous to the so-called ‘shunt’ pathway in cytochrome P450 monooxygenases,7 we have studied several flavoproteins, a.o. phenylacetone monooxygenase, for oxygenation activity when using hydrogen peroxide.9 However, we could never observe any oxygenating activity using this approach. In order to develop a flavoprotein monooxygenase that operates independent from NAD(P)H, we then focused at flavin cofactor redesign.

Scheme 1 Mechanism of flavin-based oxygenations. The solid arrows indicate the mechanism operating in natural flavoprotein monooxygenases, the dashed arrow indicates the ‘shunt’ pathway explored in this study by employing alkylated flavins 2–6.

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†Electronic supplementary information (ESI) available: All experimental procedures as well as synthesis and characterization of flavins 2–6; UV-vis of free and bound compound 3 and NMR spectra are included. See DOI: 10.1039/c1cc14039f
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For the elucidating mechanistic details of flavoenzymes and to explore flavin-based catalysis, numerous flavin derivatives have been prepared and studied in the last few decades. From these studies, it has become clear that, while natural flavin cofactors are unstable in their peroxy form, N5-alkylated flavins can form remarkably stable 4a-peroxyflavins upon reaction with hydrogen peroxide. This was first shown by Bruce and co-workers by using alkylated lumiflavins. Subsequent studies have shown that similar N-alkylated flavins are powerful oxidizing catalysts.

Inspired by these findings, we selected riboflavin-binding protein (RfBP) from Gallus gallus (chicken), which has no catalytic activity as such, but it has the capacity to bind avidly riboflavin (1, vitamin B2). RfBP serves as a riboflavin storage protein and can be easily isolated from eggs while established protocols are available to prepare the protein devoid of 1. Interestingly, it has been shown that this apo form of RfBP binds a wide range of flavin derivatives in the nanomolar range. For the elucidating mechanistic details of flavoenzymes and to provide a rationale for this, all N-alkylated flavin derivatives were prepared (Scheme 2). Alongside, apo RfBP was prepared and reconstituted with flavin derivatives. Incorporation of the reduced forms of these flavins into RfBP was successful in all cases. This could be concluded from visual inspection as, upon reconstituting the protein with an alkylated flavin derivative and excessive dialysis, the inspection as, upon reconstituting the protein with an alkylated flavin derivative and excessive dialysis, the protein remained coloured. Additionally, the binding of flavin 2 was determined by fluorescence titrations which confirmed tight binding, the derived synthetic flavoproteins were active as catalysts and even displayed enantioselectivity. Using 5S-ethylated riboflavin (flavin 2) bound to RfBP, all tested sulfides could be converted (37–55% conversion), yielding the (R)-sulfoxides in excess (10–30% ee).

Interestingly, the use of the three N-alkylated lumiflavin derivatives, flavins 3–5, yielded similar yields and optical purities of sulfoxides but, in most cases, an excess of the (S)-enantiomers. When using the demethylated form of flavin 4 (1,3,5-triethylalloxazine, flavin 6), again a competent peroxide-driven monooxygenase is formed which forms preferentially (R)-sulfoxides. In all reactions the amount of sulfone formed was very limited (≤3). For all artificial flavoenzymes, the best enantioselectivities were obtained for the aromatic sulfides. Furthermore, the results indicate that by increasing the size of the alkyl moieties of the modified lumiflavins the enantioselectivity is decreased.

As a control, the reactions were performed with each flavin derivative in the absence of apo RfBP. Without exception, this led to the formation of racemic sulfoxide (see ESIS and entry 18). Progress analysis of the oxidation reaction by RfBP complexed with flavin 3 revealed that the enantioselective outcome was best in the early phase of conversion. This appears to be due to the chemical oxidation of sulfides by hydrogen peroxide while the flavin-mediated oxidation gradually decreases in time. Finally, the oxygenating activity of 1-RfBP with hydrogen peroxide was studied, as shown in entry 19, but expectedly the results were similar to the background reaction using only hydrogen peroxide (entry 18). Concluding, the results clearly demonstrate that RfBP reconstituted with artificial flavin acts as a hydrogen-peroxide driven enantioselective monooxygenase.

Intriguingly, the observed enantioselectivity of the created flavo-enzymes is strongly dependent on the type of flavin cofactor employed. This is a very attractive feature as it allows the selective preparation of both enantiomers of the product just by changing the type of flavin cofactor. In order to provide a rationale for this, all N5-alkylated flavin derivatives studied were docked in the crystal structure of RfBP (Fig. 1). This in silico study provides insight in the origin of the enantioselectivity and cofactor modifications that steer this.

Flavin 2 was found to be bound very similar to 1. The binding is for a large part achieved by stacking of the isoalloxazine moiety.

Table 1 Enantioselective peroxide-driven sulfoxidation of prochiral sulfide by employing RfBP complexed with modified flavins

<table>
<thead>
<tr>
<th>Entry</th>
<th>R₁</th>
<th>R₂</th>
<th>Flavin</th>
<th>Protein</th>
<th>Conv. (%)</th>
<th>Ee (%)</th>
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Note: Table report % conversion and % ee. 5.0 mM substrate, 50 μM RfBP catalyst, reaction time 24 h. Determined by GC. Measured by GC or HPLC.
between tyrosine and tryptophan residues. This π–π stacking interaction is also present in the other docked flavins. However, surprisingly, flavins 3–5 bind in such a way that the alloxazine moiety is rotated by 180°. In this way, these flavins are positioned with the N5-alkyl group in a pocket that is normally occupied by the ribityl chain of riboflavin. Such an inverse binding mode may well explain the observed opposite enantioselectivity. Yet another mode of binding is predicted for flavin 6. In this case, the pyrimidine moiety of the alloxazine ring is pointing inwards while the N5-ethyl group still occupies the same binding pocket as observed for flavin 2. This is in line with the observed similarity in enantioselectivities for flavins 2 and 6 (Table 1).

This study demonstrates that by cofactor redesign, novel flavin-containing biocatalysts can be generated. By introducing peroxide-reactive flavins into a protein, it becomes catalytically active while the environment of the flavin binding cavity renders the protein selective in substrate binding, thus creating a fully self-sufficient artificial flavoprotein monoxygenase. It is worth noting that this approach of enzyme engineering does not depend on the availability of an active enzyme as a starting point. RfBP, with riboflavin bound, does not display any catalytic activity. Replacing the natural riboflavin cofactor by a mildly modified riboflavin (N5-ethylriboflavin) was found to be enough to create a peroxide-driven oxidative catalyst. Both components of such artificial flavoenzymes are relatively easy to obtain: RfBP can be obtained from chicken eggs in huge quantities while riboflavin is available on an industrial scale. It is important to note that altering the enantioselective behaviour of the biocatalyst is dependent on the employed flavin derivative. By this it was possible to obtain both enantiomers of a set of sulfoxides, albeit with moderate optical purities values. As has been shown for other artificial enzymes, e.g. artificial metallo-enzymes, tailoring by protein engineering is typically required for achieving highly (enantio)selective biocatalysts. Unfortunately, this is currently not an option when using RfBP as host protein because this protein cannot be produced in a recombinant manner. Because there are hardly any other proteins known that bind effectively riboflavin, we are currently exploring proteins that bind the phosphorylated form of riboflavin, FMN. A plethora of FMN-containing enzymes is known, providing a good starting point for design of new flavoenzymes by cofactor redesign.

Notes and references

19. In order to vary the RfBP properties, we have explored RfBP from *Coturnix japonica* (Japanese quail) because the protein sequence of the respective RfBP suggests several amino acid substitutions close to the flavin binding pocket. Unfortunately, this did not yield better results (see ESi).