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Expanding the biocatalytic toolbox of flavoprotein monooxygenases from *Rhodococcus jostii* RHA1

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ABSTRACT

With the aim to enlarge the set of available flavoprotein monooxygenases, we have cloned 8 unexplored genes from *Rhodococcus jostii* RHA1 that were predicted to encode class B flavoprotein monooxygenases. Each monooxygenase can be expressed as soluble protein and has been tested for conversion of sulfides and ketones. Not only enantioselective sulfoxidations, but also enantioselective Baeyer–Villiger oxidations could be performed with this set of monooxygenases. Interestingly, in contrast to known class B flavoprotein monooxygenases, all studied biocatalysts showed no nicotinamide coenzyme preference. This feature coincides with the fact that the respective sequences appear to form a discrete group of sequence related proteins, distinct from the known class B flavoprotein monooxygenases subclasses: the so-called flavin-containing monooxygenases (FMOs), *N*-hydroxylating monooxygenases (NMOs) and Type I Baeyer–Villiger monooxygenases (BVMOs). Taken together, these data reveal the existence of a new subclass of class B flavoprotein monooxygenases, which we coined as Type II FMOs, that can perform Baeyer–Villiger oxidations and accept both NADPH and NADH as coenzyme. The uncovered biocatalytic properties of the studied Type II FMOs make this newly recognized subclass of monooxygenases of potential interest for biocatalytic applications.

1. Introduction

Flavoprotein monooxygenases are attracting attention as selective and oxidative biocatalysts that can be used for the production of high-value chemical building blocks or pharmaceuticals [1]. These biocatalysts efficiently catalyze chemo-, regio-, and/or enantioselective oxygenations using dioxygen as mild oxidant while using NAD(P)H as reductant. Flavoprotein monooxygenases can be divided into 6 distinct classes, with each class containing sequence- and structure-related monooxygenases. Two of these classes (class A and B) are especially appealing when considering biocatalysis. These two classes are typified by being single component enzymes that contain a tightly bound flavin cofactor, while the other monooxygenases rely on multiple protomers and often employ a loosely bound flavin cofactor. The class A of flavoprotein monooxygenases seems to have evolved to catalyze aromatic hydroxylations, as most of the characterized monooxygenases of this class represent hydroxylases, typically acting on a very restricted number of substrates [2]. Class B flavoprotein monooxygenases do not catalyze hydroxylations but perform Baeyer–Villiger oxidations and/or oxygenations of heteroatom-containing compounds. In fact, three class B flavoprotein monooxygenase subclasses have been identified based on specific sequence motifs, which coincide with a preference for specific oxygenation types for each subclass [3]:

1. Baeyer–Villiger monooxygenases (BVMOs) contain the sequence motif (FxGxxxHxxxWP/D) and primarily catalyze Baeyer–Villiger oxidations, while they are also able to oxygenate heteroatom-containing compounds (N, S, B or Se containing compounds);
2. The so-called flavin-containing monooxygenases (FMOs) contain a slightly different sequence motif (FxGxxxHxxxYK/R), and are specialized in oxidizing heteroatom-containing compounds while they are inefficient in catalyzing Baeyer–Villiger oxidations. The FMOs have mainly been studied as xenobiotic degrading enzymes that help the human body to dispose toxic compounds [4,5]. The human proteome encompasses six FMO isoforms [6], which are able to activate or degrade many drugs. Only very recently FMOs have been considered for their use as biocatalysts, due to the identification and production of a microbial FMO which, in contrast to the human homologs, can be easily expressed as a soluble protein [7].
3. *N*-hydroxylating monooxygenases (NMOs) share sequence homology with the above-mentioned class B flavoprotein

Abbreviations: CE, cell extract; CCE, cleared cell extract; FMOs, flavin-containing monooxygenases; NMOs, *N*-hydroxylating monooxygenases; BVMOs, Baeyer–Villiger monooxygenases.

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monooxygenases but lack a typifying sequence motif. Only a conserved histidine can be identified in the region of the BVMO/FMO sequence motif. So far only a few NMOs from bacteria and fungi have been reported. They typically convert long-chain primary amines by *N*-hydroxylation [8].

The overall sequence homology among all class B flavoprotein monooxygenases reflects the fact that they are all single-component FAD-containing monooxygenases composed of two dinucleotide binding domains (Rossman folds to bind both FAD and NADPH), that allow them to combine flavin reduction and monooxygenation in one polypeptide chain. They often prefer the use of NADPH as electron donor, keeping the $NADP^+$ tightly bound throughout the catalytic cycle [9,10]. From the three subclasses, mainly the BVMOs have been extensively studied as biocatalysts.

To tap the natural diversity for the discovery of novel oxygenating enzymes *Rhodococcus jostii* RHA1 is a very promising candidate. The proteome of this bacterium is predicted to contain an unusually high variety of oxidative enzymes [11,12]. Inspired by this observation, we and the Grogan group have recently cloned 22 putative BVMO-encoding genes, and succeeded in producing and exploring the biocatalytic properties of these enzymes [13,14]. This research has confirmed that all these enzymes indeed act as BVMOs which can be used for a large number of oxygenations. When screening the predicted proteome of *R. jostii* RHA1 for monooxygenases, we also identified a relatively large number of other putative class B monooxygenases that seem to be more closely related to NMOs and FMOs. While BVMOs are relatively rare enzymes that are only found in bacteria and fungi with an average distribution of only one or two BVMO-encoding genes per microbial genome [15], FMOs and NMOs are even more scarce in microbes. FMOs are quite abundantly present in higher eukaryotes (e.g. the 6 isoforms in the human genome and often more than 10 in plant genomes [16]), but these FMOs are often difficult to produce due to their membrane association.

In this paper, we report on the exploration of 8 novel class B flavoprotein monooxygenases obtained from a single microorganism, *R. jostii* RHA1, that share sequence homology with FMOs and NMOs. By optimizing expression vectors and expression conditions, all enzymes were obtained in soluble and holo form. A set of ketones and aromatic sulfides was tested for all enzymes to explore their biocatalytic potential. Also their coenzyme specificity and enantioselective properties were analyzed.

2. Experimental

2.1. General materials and methods

Oligonucleotide primers were purchased from Sigma, dNTPs and In-Fusion™ 2.0 CF Dry-Down PCR Cloning Kit from Clontech, Phusion polymerase from Finnzymes. All other chemicals were obtained from Acros Organics, ABCR, Sigma–Aldrich, TCI Europe, and Roche Diagnostics GmbH. The nicotinamide coenzymes were purchased from Codexis.

The NCBI server (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was used for DNA sequence retrieval and BLAST searches. The EBI server (http://www.ebi.ac.uk/Tools/msa/clustalw2/) was used for multiple sequence alignment by CLUSTALW [19]. Treeview software (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html) was used for visualization of the sequence relationships.

2.2. Cloning and expression

Escherichia coli TOP10 from Invitrogen was used as a host for DNA manipulations and protein expression. Two expression vectors have been used: (i) a modified pBAD vector (pBAD*N*) in which the NdeI site was replaced by the original NcoI site [17], and (ii) the recently engineered pBAD-based pCRE2 vector which harbors a codon-optimized gene encoding a thermostable mutant of phosphite dehydrogenase (PTDH) with an N-terminal His-tag [18].

The target genes were amplified by PCR using genomic DNA of *R. jostii* RHA1 as template and subsequently cloned into pBAD*N* using the In-Fusion PCR Cloning kit from Clontech, following the recommendations of the manufacturer. Expression of all generated expression constructs was tested using 24-multiwell microtiter plates in the sandwich cover system from Enzyscreen®. Cell cultures of 2.5 mL in LB medium supplemented with 50 $\mu g\,mL^{-1}$ ampicillin were grown at four different temperatures (17 °C for 48 h; 24 °C for 32 h; 30 °C and 37 °C for 16 h) with four different arabinose concentrations (0.002%, 0.02%, 0.2%, and none) each, with shaking at 200 rpm. Cell extracts (CEs) were obtained using DNase/lysozyme in combination with freezing in liquid nitrogen and thawing at 30 °C. CEs as well as cell cleared extracts (CCEs) were analyzed by SDS-PAGE for (soluble) expression of the monooxygenases. For the genes that did not yield expressed soluble protein when cloned into pBAD*N*, the in-house developed pCRE2 expression vector was used.

2.3. General procedure for the bioconversions employing the novel FMOs

Conversions were performed essentially as described before [14]. For GC analysis, 500 μL incubations of 50 mM Tris/HCl pH 7.5, 10% glycerol, 1 mM DTT, 1 mM EDTA, 10 μM FAD, 5 mM substrate, 5% cosolvent (1,4-dioxane), 100 μM NADPH, 3.1 μM PTDH, 10 mM phosphite and 5 μM of the corresponding monooxygenase in CCE form (total volume of 0.5 mL), were shaken in glass vials at 24 °C for 24 h. For determining the exact concentration of each enzyme in the respective extract, a recently developed method was used which relies on the decrease in absorbance at 450 nm upon NADPH-mediated reduction of the flavin cofactor [14]. Each conversion was stopped by extracting with ethyl acetate (2 × 0.5 mL containing 0.1% mesitylene as standard), dried with magnesium sulfate and analyzed directly by GC to determine the degree of conversion and the enantioselectivity. The details concerning the (chiral) GC analysis can be found in the Supplemental information (Table S1). For every tested reaction, control experiments in the absence of enzyme resulted in no conversion.

3. Results

3.1. Identification of putative FMO/NMO-encoding genes

By a PBLAST search of the proteome of *R. jostii* RHA1 [11], not only 23 Type I BVMO-encoding genes could be found [14], but also another 8 genes putatively encoding class B flavoprotein monooxygenases could be identified. All corresponding proteins contain two typical Rossman fold motifs (GxGxxG), clearly distinguishing them from other flavoprotein monooxygenase classes. As for all class B flavoprotein monooxygenases, one Rossman fold motif is close to the N-terminus while the other is in the middle of the sequences. In our previous work [14], we proposed a new conserved sequence motif typical for the Type I BVMO family: [A/G]GxWxxxx[F/Y]P[G/M]xxxD located between the two Rossman fold motifs. This motif entails the conserved active site aspartate and therefore appears a better fingerprint for Type I BVMO sequences. The motif is absent in all sequences used in this study and confirms that they do not represent classical BVMOs. The sequences do also not contain the previously described BVMO-typifying motif. In fact, most of them (all except for monooxygenase