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*Vp*StyA1 and *Vp*StyA2B of *Variovorax paradoxus* EPS: Rather an Aryl Alkyl Sulfoxidase than a Styrene Epoxidizing Monooxygenase

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Abstract: *Vp*StyA1 and *Vp*StyA2B of *Variovorax paradoxus* EPS is annotated and characterized as the first representative of an E2-type styrene monooxygenase of proteobacteria. It comprises a single epoxidase (*Vp*StyA1) and a fusion protein (*Vp*StyA2B) which serves mainly as NADH:FAD-oxidoreductase. *Vp*StyA2B had a K_m of 33.6 ± 4.0 µM for FAD and a k_{cat} of 22.3 ± 1.1 s^{-1}. *Vp*StyA2B and *Vp*StyA1 showed monooxygenase activity on styrene of 0.14 U mg^{-1} and 0.46 U mg^{-1} as well as on benzyl methyl sulfide of 1.62 U mg^{-1} and of 3.11 U mg^{-1}. A putative fusion region at position 408 (AREAV) was mutated to provide insights on *Vp*StyA2B-function. The best mutant (408-AAAAA) obtained showed a 6.6-times higher affinity for FAD while keeping the NADH-affinity and -oxidation activity. Corresponding epoxidase activity increased (1.6-times). But, other mutants showed still NADH:FAD-oxidoreductase activity, but lost mostly their epoxidase activity indicating effects on the monooxygenase-part as well. Thus, this monooxygenase system represents an interesting candidate for biocatalyst development.

Keywords: sulfoxidation; epoxidation; two-component monooxygenase; flavoprotein; enantioselective biotransformation; fusion protein; protein linker; soil microorganism

1. Introduction

Flavin-dependent monooxygenases are able to catalyze a number of biotechnological important reactions which are often regio- and enantioselective [1-3]. These enzymes can be divided into eight groups according to their structure and function. For many purposes selective epoxidation and sulfoxidation became attractive [4-11] and among those flavin-dependent styrene monooxygenases (SMOs) exist as a group performing both reactions with a certain selectivity [2,5-10]. This is group E among the flavin-dependent monooxygenases which can be described as follows (EC 1.14.14.11). It is a two-component system at which a strictly NADH-dependent reductase (StyB; EC 1.5.1.36) produces reduced FAD for the monooxygenase component (StyA, StyA1). Some of these reductases occur as self-sufficient fusion proteins of an oxygenase and reductase domain (StyA2B) and those represent the prototype of E2 like SMOs which was first described from a *Rhodococcus* [5]. Most of the monooxygenases (StyA, StyA1) described so far utilize styrene as major substrate and belong even to a natural styrene degradation pathway [11]. Recently, some E2-type SMOs have been discussed as initial part of indole detoxification or degradation [12,13]. Thus they can also be described as indole monooxygenases, IMOs.

SMOs rely on the flavin cofactor flavin adenine dinucleotide (FAD). The reduced flavin is transferred by diffusion or by a direct transfer between both components [14-16]. The

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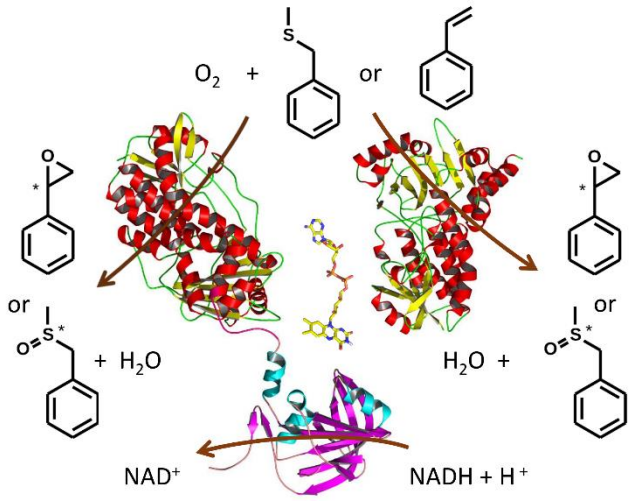
monooxygenase tightly binds the reduced cofactor and the oxygen driven catalysis gets initiated [14-18]. Here oxygen gets activated by the reduced flavin to a (hydro)peroxy-FAD which can attack the actual substrate. Upon substrate oxygenation a hydroxyl-FAD intermediate is formed and decomposes to oxidized FAD and water [17-19]. The product is subsequently released and another catalytic cycle can start.

Styrene monooxygenases of type E2 have been shown to be excellent in sulfoxidation with respect to substrate conversion and enantioselectivity whereas the E1 type only shows a high activity at low enantioselectivity [1-10]. However, so far there is only a single E2 type SMO in detail studied and it has a comparable low activity [5,6,9]. Thus, it was reasonable to screen for further E2 type SMO candidates which can be applied in biocatalysis.

Powered by our former studies on E2 type SMOs [4-6,9,11,20,21] we took the chance to investigate the phylogenetic more distinct system *Vp*StyA1/*Vp*StyA2B of *Variovorax paradoxus* EPS (accession numbers: ADU39063 and ADU39062). Thus we here present its general activity, capability to act on styrene but also on sulfides (Scheme 1) compared to other monooxygenases and to evaluate its biotechnological applicability.



Scheme 1. Cartoon of styrene monooxygenase activity. The reductase domain of StyA2B reduces FAD (displayed in oxidized form between protein monomers) upon NADH-consumption. Reduced FAD can be used by both monooxygenase units StyA1 (major; right) and StyA2 (minor; left) to activate molecular oxygen and to subsequently oxygenate the substrates (here for example styrene and benzyl methyl sulfide) [5-10]. Upon substrate oxygenation hydroxyl-FAD is formed and thereof water is eliminated to recycle the FAD in its oxidized form for the next catalytic cycle.

2. Results and Discussion

2.1. Evolution of VpStyA1 and VpStyA2B from strain EPS

Already during an earlier study, the putative genes encoding for *Vp*StyA1 and *Vp*StyA2B and thus the respective gene cluster of strain EPS had been identified [20]. According to a phylogenetic analysis and the surrounding genomic region the proteins were assigned as SMO related to the E2 prototype of *Rhodococcus opacus* 1CP [5,6,11]. Interestingly, the sequence similarity of *Vp*StyA1 and *Vp*StyA2B (74 % identity over 404 amino acids) between both monooxygenase subunits was much higher as among other StyA1/StyA2B couples. Further, they form together a separate branch in a distance tree of monooxygenase components [20,22]. As in the meanwhile more of those putative sequences had been released we could refine the phylogenetic study and try to understand the fusion even in more detail (Fig. 1). Respectively, the fusion event was discussed as functionally convergent event which is reinforced by the updated phylogenetic distance tree. However, no activity of these putative SMOs of type E2 originating of *Variovorax* species has been reported until now!

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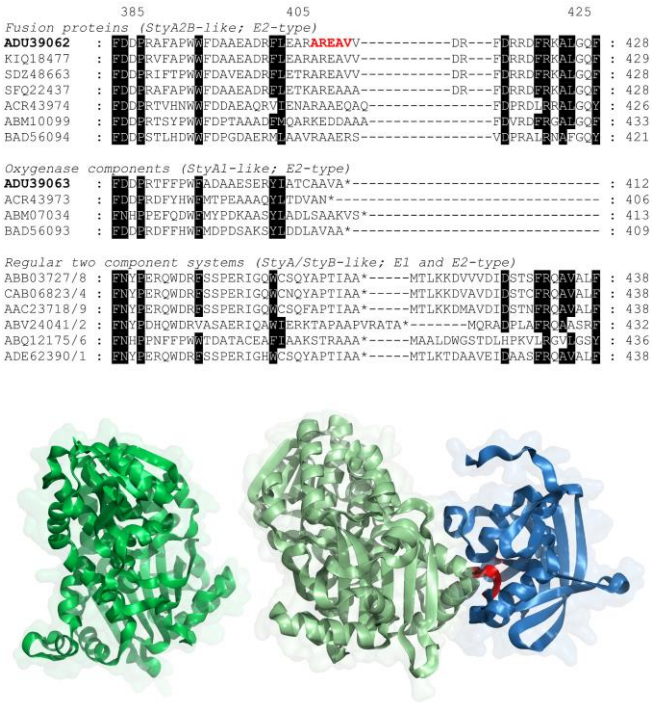

Figure 1. (Top) Sequence alignment of StyA2B-like proteins in comparison to functional StyA and StyB components. (Bottom) homology model of *Vp*StyA1 (darkgreen) *Vp*StyA2B (monooxygenase – lightgreen, reductase – blue). The proposed linker region is illustrated in red. Numbering on top is according to *Vp*StyA2B and the terminal amino acids are given at the end of lines. (ADU_*V. paradoxus* EPS; KIQ_*V. paradoxus* MEDvA23; SDZ_*Variovorax* sp. YR266; SFQ_*Variovorax* sp. OK605; ACR_*R. opacus* 1CP; ABM_*Paenarthrobacter aurescens* TC1; BAD_*Nocardia farcinica* IFM 10152; ABB_*Pseudomonas putida* SN1; CAB_*P. fluorescens* ST; AAC_*P. taiwanensis* VLB120; ABV_uncultured bacterium; ABQ_uncultured bacterium/MoxY; ADE_*Pseudomonas* sp. LQ26).

Mining the databases for *styA2B*-like genes/proteins revealed that they occur mainly among genera of Actinobacteria (e.g. *Amycolatopsis*, *Arthrobacter*, *Gordonia*, *Mycobacterium*, *Nocardia*, *Paenarthrobacter*, *Paeniglutamicibacter*, *Pseudarthrobacter*, *Sciscionella*, *Sinomonas*, *Streptomyces*, among others) but also among *Variovorax* (date of BLAST search: 19th Sep. 2017). So far there are no *styA2B*-like genes found in other none-Actinobacteria. However, there exist other *styA*/*styB* genes closely related to this subtype E2 of SMOs in various genera. The closest homologue to *Variovorax* SMOs are found in *Delftia* although no fusion variant is present. Already with the first report of *Ro*StyA2B the linker region was recognized, but not investigated [5]. This we now did by mean of sequence alignments as well as molecular modelling (Fig. 1). And we identified a less conserved region/loop at the *C*-terminal site of the monooxygenase domain components of StyA2B-like proteins as potential linker region. In case of *Vp*StyA2B it is located around region 408-AREAV-412. From the alignment of StyA2B-, StyA1- and StyA-like sequences it gets clear that the last conserved amino acid among those proteins is at position 404 (*Vp*StyA2B) and in case of the none-fused proteins the following *C*-terminal parts are variable. In case of the fusion proteins next to this region the domain of the NADH:FAD oxidoreductase (B-part) is localized. There the first conserved amino acid is at position 417 (*Vp*StyA2B) with respect to related StyA2B- and StyB-like proteins. This region from 404 to 417 represents a flexible loop between two helices according to the models obtained from homology modelling. This is not surprising as there is no sequence-structure relation available for this part. However, it fits to earlier made observations in which the start of the reductase domain was investigated [9,19,20,23]. Respectively, this proposed linker region need to be investigated.

Adjacent to the monooxygenase domain and the proposed linker region the reductase domain of the fusion proteins follows. Interestingly, the reductase sequence misses in all cases a few amino acids (range: 3 to 14) when compared to the StyB reductases of E1 type SMOs (Fig. 1) [5,7]. The

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mentioned linker region was now target for a subsequent site-directed mutagenesis investigation. In all cases the original sequence (408-AREAV) was replaced or even extended. The following mutants have successfully been prepared and verified by sequencing: 408-TIVVV, 408-AAAAA, 408-HHHHH, 408-WYHHH, 408-WYHHHHH, and 408-GQWCSQY. In order to validate the made assumptions, the wildtype protein *Vp*StyA2B and the mutant proteins were produced and assayed. The chosen linker sequences of mutants to be produced base on the following rationale. Linker with A- and V-rich sequences were chosen to be flexible or H-rich to be more rigid. Whereas, the WY-H-variants should represent bulkier and thus even more restricted linkers. The 408-GQWCSQY motif represents a terminal part of a StyA-protein originating of pseudomonads (Fig. 1) and was picked as a comparison with respect to earlier made fusion proteins [19].

2.2. Molecular genetic work and enzyme production

The cloning of both genes, *VpstyA1* and *VpstyA2B*, as well as the generation of mutants was successfully accomplished which had been verified by sequencing the inserts of gene expression plasmids as described in experimental section (See supporting information, Table S1) and by a simple activity assay. Since *E. coli* BL21 derivatives allow the formation of indole from tryptophan during growth on complex medium as used herein, the activity of styrene monooxygenases and related enzymes can be verified by indole transformation to indigo [4-6,10,11,19]. And indeed, all clones obtained (*E. coli* BL21 (DE3) pLysS derivatives harboring the wildtype or mutant genes in a pET-vector) produced indigo during cultivation even without being induced for overexpression of target proteins. Clones which showed highest rate in indigo formation had been selected, propagated and stored as glycerol stocks for later protein production and characterization efforts.

Gene expression and protein purification was performed as described elsewhere [9]. The individual clones were cultivated either in a 3 L-fermenter or in 1 L-flasks in LB medium with appropriate antibiotics and 0.1 mM IPTG as inductor as described previously. In both cases (*Vp*StyA1 and *Vp*StyA2B), enzyme production was successfully achieved with a yield of 2 to 4 $mg_{VpStyA2B}$ and up to 9 $mg_{VpStyA1}$ protein per liter broth, respectively. This is in congruence to other studies. Subsequently, the proteins produced and purified were characterized.

2.3. Reductase activity of VpStyA2B and mutants

The fusion protein *Vp*StyA2B of strain EPS was supposed to be rather a reductase of the complete SMO system [6,7], and was for those reasons in analogy to the enzyme of strain 1CP characterized.

The protein *Vp*StyA2B was successfully produced (verified by SDS-PAGE, not shown). The fraction obtained from Ni-chelate chromatography was slightly yellow which is an indicator for a bound flavin. This was analyzed as described for other SMOs and FAD was clearly determined by means of RP-HPLC as well as by spectroscopic methods and the use of authentic standards. A FAD saturation of 4.9 to 20.8 mol-% was calculated for the protein applied. This is in congruence to results obtained earlier for *Ro*StyA2B [6,17]. This fraction was immediately assayed for activity and then concentrated and stored at -20 °C in a suitable storage buffer until characterization. Indeed, it could use NADH as source for reducing equivalents in order to reduce flavins. NADH cannot be replaced by NADPH. But, in case of the flavins FAD, FMN and riboflavin can be acceptors of reducing equivalents (Table 1). However, a clear preference could not be determined with respect to catalytic efficiency, which was for all here employed flavins between 0.57 and 0.88 s^{-1} μM^{-1}, respectively.

Table 1. NADH:flavin oxidoreductase activity of *Vp*StyA2B (MW = 66.32 kDa which was calculated from the amino acid sequence including the *N*-terminal tag).

Donor [1] / Acceptor [μM] [2]	K_m [μM]	V_{max} [U mg^{-1}]	k_{cat} [s^{-1}]	k_{cat} K_m^{-1} [s^{-1} μM^{-1}]

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NADH (7.9 - 164) / FAD (70)	24.0 ± 4.0	16.2 ± 0.8	17.9 ± 0.9	0.72
NADH (164) / FAD (6.3 - 78.8)	33.6 ± 4.0	20.2 ± 1.0	22.3 ± 1.1	0.64
NADH (164) / FMN (4.1 - 90.2)	45.9 ± 6.8	26.0 ± 1.8	28.7 ± 1.9	0.57
NADH (164 µM) / Riboflavin (6.3 - 88.2)	37.7 ± 7.2	31.3 ± 2.7	34.6 ± 2.9	0.88

[1] NADPH (230 µM in presence of 70 µM FAD) did not serve as an electron donor.

[2] Either electron donor or acceptor was present in excess and the data obtained of triplicates were analyzed assuming Michaelis-Menten kinetics.

The reductase activity and catalytic efficiency of *Vp*StyA2B is up to 10-times higher than reported for the *Rhodococcus* enzyme *Ro*StyA2B [5]. Thus, differences on amino acid level (57 % identity) are reflected within the biochemical properties of StyA2B-like SMOs. Still, the activity of the fusion protein is by an order of magnitude lower if compared to most reductases of two-component systems [22-24]. However, it was reported recently that the *N*-terminal fusion to the monooxygenase drastically decreases the oxidoreductase activity in RoStyA2B [23]. In addition, an artificial fusion protein was constructed from two-component relatives of *Pseudomonas* [19]. Herein, the coupling was improved and the catalytic mechanism changed. It was also shown that the *N*-terminal region of the reductase influences the binding and affinity for the substrate [19,22]. Therefore, it is likely that the same is true for *Vp*StyA2B. However, it is likely that this effect of the fusion to StyA2 is different in dependence of the linker region in the *Variovorax* enzyme.

In contrast to the monooxygenase part (StyA2), promiscuity towards the flavin cosubstrate is in accordance with most characterized SMO reductases. And so far, only one representative from strain 1CP (*Ro*StyB) is reported to be selective for FAD [9,22-24].

*Vp*StyA2B was tested for inhibition or activation by a number of compounds used in other SMO studies as well. Concentrations were adjusted to those of other studies or relevant for herein performed enzyme assays. The NADH:FAD oxidoreductase test was employed. Therefore, the enzyme was pre-incubated with a compound for 10 min at 20 °C. And the following relative activities compared to the wildtype enzyme were determined (Fig. S1): a) No effect was observed for styrene (2 mM), styrene oxide (100 µM), methyl phenyl sulfoxide (200 µM), H_2O_2 (100 µM), iodoacetamide (2 mM), sodium azide (500 µM) and Mg^{2+} (20 µM). b) A decrease in effect was determined in presence of styrene oxide (200 µM; 87 % rel.-activity remained), methyl phenyl sulfide (2 mM; 24%), EDTA (1 mM; 88%), Fe^{2+} (100 µM; 74%), Fe^{3+} (100 µM; 65%), Ni^{2+} (100 µM; 81%), Zn^{2+} (100 µM; 35%), Ag^{+} (10 µM; 0%), Cd^{2+} (10 µM; 15%) and Hg^{+} (10 µM; 25%). c) And an increased activity was determined for o-phenantroline (1 mM; 132%), DTT (500 µM; 186%), mercaptoethanol (500 µM; 166%), Ca^{2+} (100 µM; 198%) and Mn^{2+} (100 µM; 165%). The effects of the herein tested compounds are similar as observed for *Ro*StyA2B [5]. Thus it can be concluded, that there is no metal dependency present. Moreover, in contrast to *Ro*StyA2B, the activity is lowered by addition of Fe-ions, while Fe^{3+} has a stronger effect. This is in accordance with inhibition studies on the reductase StyB from the two-component systems of *Pseudomonas taiwanensis* VLB120 and *Acinetobacter baylyi* ADP1 [22,24]. Here, the effect of Fe^{2+} is even stronger. However, reducing agents as DTT and divalent ions as Ca^{2+} support the performance of *Vp*StyA2B. This was also observed for *Ro*StyA2B as well as *Ab*StyB although the mechanism is likely different. In contrast to *Ro*StyA2B, *Vp*StyA2B and *Ab*StyB are insensitive against hydrogen peroxide and the protective effect might therefore not be attribute towards oxidation of cysteine residues. It was rather proposed to be a masking of Ni^{2+}-ion that impeded the reductase [22]. However, this is unlikely as two additional purification steps were applied for the *Vp*StyA2B preparation and majority of the Ni^{2+} should be removed. Interestingly, thioanisole decreases the reductase activity, which was not observed for SMO-reductases before.

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In order to investigate the fusion region of this protein affords to determine the respective region had been accomplished on sequence level (see above) and served for the direction in a mutagenesis study. The six mutants of *Vp*StyA2B obtained were separately produced and purified as described above and in analogy to the wildtype characterized for their NADH:FAD oxidoreductase activities (Table 2). During expression also these clones produced significant amounts of indigo indicating a functional expression of respective SMO-variants.

Table 2. NADH:flavin oxidoreductase activity of *Vp*StyA2B-mutants and -wildtype.

*Vp*StyA2B-variant	Donor NADH [1]		Acceptor FAD [1]	
	K_m [μM]	V_{max} [U mg^{-1}]	K_m [μM]	V_{max} [U mg^{-1}]
wildtype *Vp*StyA2B	24.0	16.2	33.6	20.2
408-TIVVV	37.7	3.3	5.1	3.1
408-AAAAA	28.1	8.9	1.8	8.8
408-HHHHH	44.2	2.5	14.5	3.1
408-WYHHH	20.1	7	3.2	6.9
408-WYHHHHH	40.6	13.8	7.4	13.2
408-GQWCSQY	47.6	3.8	14.5	3.7

[1] Concentrations of NADH and FAD in order to determine kinetic properties were chosen as for the wildtype *Vp*StyA2B given in Table 1. As previously triplicates were used to get the values and standard deviations were in all cases less than 15 % and thus comparable to Table 1.

The mutants were all active and it was possible to determine kinetic parameters as for the wildtype oxidoreductase. Respectively, the wildtype was most active with NADH and all the mutants showed a lower activity. However, the affinity for NADH seemed not to be altered as the K_m values were all in the same high range. This picture changed somewhat for the series collected for FAD. The general activities were similar to those for NADH-variation and also the order from the most active to the worst representative were same. But, the affinity for FAD was drastically different among the variants which was obvious from the K_m values determined. Thus, the best and most efficient variant was 408-AAAAA with respect to its oxidoreductase activity. Respectively, the catalytic efficiency of FAD-reduction is about 5.4 s^{-1} μM^{-1} which is about 8.4-times more efficient than the wildtype. This is due to the tighter FAD binding expressed by the 6.6-times lower K_m value for FAD. Thus means the proposed linker region, which was mutated, has a strong effect on the FAD binding and turnover. This is in congruence to earlier studies which could demonstrate that the *N*-terminal part of NAD(P)H:flavin oxidoreductases is important for the flavin binding and thus for catalysis [19,23]. Further, it indicates that the selected region indeed can be the linker as it is functional relevant for the reductase domain.

2.4. Monooxygenase activity of VpStyA1, VpStyA2B and VpStyA2B-mutants

Already during the gene cloning and expression experiments the formation of indigo was observed and used to identify best protein producers as mentioned above. This was later verified by a plate assay as described in experimental section in order to get a view on the strains producing either a highly active SMO or a lot of this SMO (Table 3). The wildtype proteins *Vp*StyA1 and *Vp*StyA2B and especially the mutant 408-AAAAA produced most indigo. The acceptance of indole as a substrate and the formation of indigo fits to a very recent finding that related E2 type monooxygenases were assigned to indole degradation and act basically as indole monooxygenases (IMOs) [12,13].

Table 3. Epoxidase activity of *Vp*StyA2B-mutants and –wildtype enzymes.

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Substrate		Styrene V_{max} [mU mg^{-1}]		Indole
SMO [1]	-	ETY [2] [%]	extra *Ro*StyBart [3]	plate screening [4]
wildtype *Vp*StyA1	n.a.	n.a.	460	+++
wildtype *Vp*StyA2B	159	1	140	++
408-TIVVV	135	4.4	327	+
408-AAAAA	260	3	260	+++
408-HHHHH	< 1	< 1	4	+
408-WYHHH	1.7	< 1	7.3	+
408-WYHHHHH	< 1	< 1	1.6	+
408-GQWCSQY	< 1	< 1	3.1	+

[1] Mutants are made of *Vp*StyA2B as described in methods and were in analogy to the wildtype investigated.

[2] The electron transfer yield (ETY) was calculated from the NADH-consumption vs. epoxidation rate.

[3] In case of StyA1 an additional NADH:FAD oxidoreductase was needed which could also assist StyA2B-like proteins [6,23].

[4] The plate screening was performed with clones expressing respective genes on an agar plate and the indigo formation was followed online by a camera.

n.a. = no activity measureable

The wildtype monooxygenases *Vp*StyA1 and *Vp*StyA2B show a comparable epoxidase activity as other SMOs (range 0.02 to 2.1 U mg^{-1}) (Table 3) [5-10,24]. *Vp*StyA1 is about 2.9-times and 2.1-times more active than *Vp*StyA2B and the *Rhodococcus* counterpart *Ro*StyA1, respectively. And it was confirmed that *Vp*StyA1 represents the major monooxygenase activity of the system as it was found for the prototype *Ro*StyA1/*Ro*StyA2B [6]. This was expected but also indicates that the high sequence similarity of the *Variovorax* monooxygenases does not change the catalytic properties to a large extent. However, it is interesting that *Vp*StyA2B is about 8.4-times more active on styrene as *Ro*StyA2B. The latter one could be boosted by additional FAD-reductase, but, *Vp*StyA2B not. Later another batch of *Vp*StyA2B was produced for biotransformations. It showed only a specific epoxidase activity of 27 mU mg^{-1} which is about 6-times lower. In this case surplus of reductase activity by an additional FAD-reductase could boost the activity 2.3-times. This indicates that *Vp*StyA2B can be saturated by reduced FAD to a certain maximum as it was found for the *Rhodococcus* counterpart [6]. Another difference between both StyA2B-proteins is the FAD-reductive power which is about 4.3-times higher in case of *Vp*StyA2B with a k_{cat} of 22.3 s^{-1} vs. that of *Ro*StyA2B of 5.2 s^{-1}. This might be the reason that *Vp*StyA2B can reach a maximum epoxidase activity even without an additional FAD-reductase (Table 1). Furthermore, the quality of the protein batches prepared can differ with respect to FAD saturation as well as activity which is known for related enzymes [5,6,9,17]. However, we could not identify the fact which led to this different specific activity as the only differences during enzyme production were the fermentation volume and a final polishing step (gel filtration). The protein batches had otherwise a similar concentration and purity after purification which was also consistent except for the volumes applied. This needs further investigation.

The mutants obtained were in analogy to the wildtype enzymes characterized for their capability to convert styrene into styrene oxide. The wildtype *Vp*StyA2B showed an activity of 159 mU mg^{-1}. The *Vp*StyA2B-variants were prepared similarly and assayed immediately in order to allow a direct comparison. The following relative styrene epoxidase activities were determined: 84.9 % for 408-TIVVV, 163.5 % for 408-AAAAA and less than 2 % in case of the other variants (408-HHHHH, 408-

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WYHHH, 408-WYHHHHH, and 408-GQWCSQY). This clearly indicates the region mutated has significant effects also on the epoxidase activity of StyA2B-proteins even if it seemed to be only the *C*-terminal part of the respective monooxygenase domain. Further, from the nature of this proposed linker sequence it can be reasoned that smaller hydrophobic residues yield higher activities compared to larger or more polar residues. This is indeed true for the FAD-reductase as well as the corresponding styrene epoxidase activity. And again the mutant 408-AAAAA showed a very promising catalytic behavior which was better than the wildtype in terms of activity and efficiency (Table 3). It was followed by variant 408-TIVVV which was even more active with surplus of reduced FAD formed by an additional FAD-reductase. It achieved almost the epoxidase activity of *Vp*StyA1. This might allow to draw some conclusions as it might be possible to generate more efficient and catalytic active self-sufficient fusion proteins; here SMO-like proteins. But, as the mutagenesis of the *C*-terminal part of the StyA2-part improved catalytic properties it might be also possible to alter StyA1- or StyA-like proteins at their *C*-terminus in order to improve their catalytic behavior. However, structural investigations become necessary in order to elucidate the nature of substrate and cofactor binding to really understand why the mutants show a distinct activity and efficiency!

2.5. Biotransformation of sulfides

For the biotransformation experiments, another fresh batch of each enzyme (*Vp*StyA1 and *Vp*StyA2B) was produced and the initial specific activity of styrene epoxidation was determined to 27 mU mg^{-1} *Vp*StyA2B, 63 mU mg^{-1} *Vp*StyA2B and surplus *Ro*StyBart as well as 121 mU mg^{-1} *Vp*StyA1 and surplus *Ro*StyBart, respectively [23]. The lower specific activities of these batches might be due to remaining impurities in the protein preparation as for these experiments after the Ni-chelate chromatography the additional gel filtration was omitted. Another important point is that the addition of extra reducing power in form of *Ro*StyBart supports the monooxygenase performance, as it was discussed above and which is in congruence with earlier studies. For comparison, in the 2 h biotransformation assay with styrene as substrate, observed activities with surplus *Ro*StyBart were 0.002 U mg^{-1} for *Vp*StyA2B and 0.029 U mg^{-1} for *Vp*StyA1.

As target compounds sulfoxides had been chosen and thus kinetics and later on biotransformations were done with selected model sulfides. Kinetic parameters were determined as described above for styrene in order to allow a comparison. Maximum activity on the substrate benzyl methyl sulfide (BMS) from such kinetic studies was determined to 3.113 U mg^{-1} *Vp*StyA1 and 1.616 U mg^{-1} *Vp*StyA2B. This is about 25 to 26-times faster as the conversion of styrene. And taking the maximal epoxidase activity of *Vp*StyA1 (Table 3) into account, a specific activity of about 10 U mg^{-1} could be possible. This high sulfoxidase activity of the SMO system of *Variovorax paradoxus* EPS is impressive and thus more sulfides were tested in a series of 2 h biotransformations (Table 4).

*Vp*StyA1 reaches observed activities of up to 0.281 U mg^{-1} and BMS is the best substrate. These activities are much higher if compared to styrene as substrate. *Vp*StyA1 produces almost exclusively the (*S*)-sulfoxide products. *Vp*StyA2B has lost about 97 % of its initial activity by up to 0.044 U mg^{-1}, but also preferably produces the (*S*)-enantiomer. However, due to the mentioned loss of activity the exact ee-determination failed.

However, both monooxygenases lost at least one order of magnitude of activity and it has to be elucidated what causes the decrease. It might be possible that there is product inhibition or that the enzymes are not stable over time under the assay conditions. Especially, the more complex fusion protein seems to be less stable in cell-free approaches.

Table 4. 2 h Biotransformation of sulfides. Activities are given in U mg^{-1} and represent the observed activities determined after 2 h (no initial rates). Conversions of 2 mM substrate are given.

Substrate	Product	*Vp*StyA1 [U mg^{-1}]	ee [%]	*Vp*StyA2B [U mg^{-1}]	ee [%]

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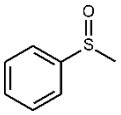
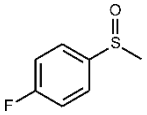
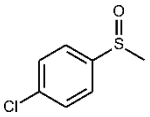
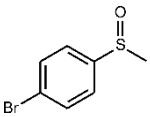
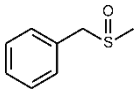
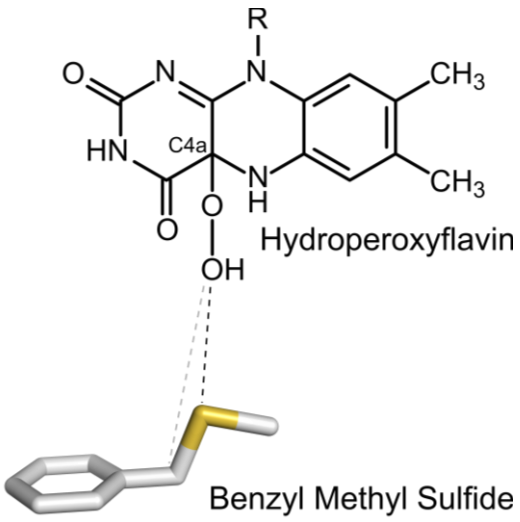
		(conversion [%])		(conversion [%])	
PMS		0.129 (43.6 ± 1.9)	98 *(S)*	0.021 (6.3 ± 0.3)	64 *(S)*
4F-PMS		0.177 (59.8 ± 0.4)	99 *(S)*	0.015 (4.3 ± 0.7)	84 *(S)*
4Cl-PMS		0.114 (38.5 ± 1.7)	99 *(S)*	0.015 (4.4 ± 0.4)	96 *(S)*
4Br-PMS		0.096 (32.4 ± 4.7)	99 *(S)*	0.021 (6 ± 0.7)	96 *(S)*
BMS		0.281 (95 ± 0.5)	97 *(S)*	0.044 (13 ± 0.9)	n.d. *(S)*

phenyl methyl sulfide (PMS), 4-fluoro phenyl methyl sulfide (4F-PMS), 4-chloro phenyl methyl sulfide (4Cl-PMS), 4-bromo phenyl methyl sulfide (4Br-PMS), benzyl methyl sulfide (BMS); n.d. = not detectable

Also it need to be mentioned that in all cases no other products had been observed which could indicate any overoxidation of sulfoxides to sulfones. This is an important feature to produce chiral sulfoxides.

When comparing the sulfide biotransformations it gets obvious that they are for each enzyme in the same order of magnitude. And in both cases a significant preference for the substrate benzyl methyl sulfide was observed. Here the S-atom is one positon more far from the aryl system as in case of the other sulfides applied. This indicates that the activated oxygen of the hydroperoxy-FAD intermediate in the active site of the monooxygenase is more closely located to this position of the substrate and allows a faster attack (Scheme 2) [17,18]. This hypothesis needs to be verified by structural and kinetic investigations. But, so far there is no experimentally proven SMO structure inclusive substrate and cofactor available.



Scheme 2. Illustration of the putative binding and positioning of benzyl methyl sulfide towards the hydroperoxyflavin in the active site of SMOs from *V. paradoxus* EPS. Kinetic data indicate that the

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distal position of the S-atom is likely closer to the C4a-hydroperoxyflavin, as BMS is preferred over sulfides, were the S-atom is adjacent to the aryl group.

3. Materials and Methods

3.1. Synthesis of sulfoxides

Commercial sulfides served as base to produce sulfoxides which had been applied as standards for analytical methods. The chemical sulfoxidation was achieved according to the protocol published earlier [25] as it provided high yields of desired products. First a solution of 7 mmol sulfide in 100 ml methanol and 20 ml water plus 10 ml titanium (III) chloride (16 % aqueous solution) was prepared. Then dropwise a hydrogen peroxide solution (3.2 ml 30 % aqueous in 15 ml methanol) was added while constantly stirring at ambient temperature (about 20 °C). Latest after 25 min the substrate was completely converted and the reaction had been stopped by adding 50 ml water. Sulfoxides formed had been extracted by chloroform (three times) and subsequently dried over anhydrous magnesium sulfate. Chloroform was further removed under reduced pressure. Success of reactions and purities of products had been determined as described elsewhere [26].

3.2. Nucleotides, sequence analysis and molecular modelling

Sequence analyses based on a previously performed investigation [5]. Thus accession numbers of the E2-type SMO components originating of *Variovorax paradoxus* EPS were already available: ADU39063 (*Vp*StyA1) and ADU39062 (*Vp*StyA2B). These were used for a BLASTP search in order to identify related or even homolog proteins. This allowed to generate an amino acid sequence alignment in analogy to earlier reports [5,20]. This served as a template for the subsequent calculation of a dendrogram and the modelling. As templates for homology modelling the two available structures (PDB StyA: 3IHM, and PDB PheA2: 1RZ0) were employed [18,27]. The *C*-terminal StyA2-part was linked to the *N*-terminal StyB-part by a random loop which gets obvious from Scheme 1 and Figure 1, respectively. As tools have been employed: MEGA7 for the alignment, Modeller program version 9.19 for the comparative homology modelling as well as GenDoc and PyMol V1.1r1 for alignment and 3D-structure visualization [9,28-30].

3.3. Bacterial strains and cultivation

Escherichia coli strains DH5α and BL21 (DE3) pLysS were cultivated for cloning and expression as described elsewhere [5,6,9,31]. A list of genes, primers and plasmids for this work is presented in the Supplemental Information (Table S1).

Indigo formation was observed during the cultivation and gene expression experiments. A plate screening in order to monitor indigo formation was set up as follows. The expression clones were transferred on a M9 mineral media agar-plate in a predefined grid, followed by a 22 h incubation at 30 °C [32]. The solid media was supplemented with antibiotics as described previously and 0.5 mM isopropyl-β-*D*-thiogalactopyranosid for induction of protein expression [5,6,9,31]. Indigo formation was started by spraying the plates with an indole-substrate solution (5 Mm Tris-HCl, pH 7.5, 50 mM indole, 20 % DMSO). The plates were then transferred onto a white light-table in a darkened box equipped with a camera. Pictures of the plates were shot in a 30 s interval. The color formation of each colony was monitored and normalized upon colony size to get a time-resolved intensity profile for each clone.

3.4. Molecular genetic work

The DNA sequences of *VpstyA1* (Accession number: MF781076) and *VpstyA2B* (MF781075) were optimized for the codon usage and GC content of *Acinetobacter baylyi* ADP1 as described previously [9,33]. The genes were purchased in a pEX-A vector system from Eurofins MWG (Ebersberg) with 5'-NdeI and 3'-NotI restriction sites allowing for subcloning into pET16bP [9]. Site-directed mutagenesis of the linker area was done by using the GeneMorph II EZClone Domain Mutagenesis Kit (Agilent

Technologies). Therefore, a megaprimer was generated by amplifying the target region with a primer that contains the desired mutation. This megaprimer was annealed to the parental plasmid and extended in the EZClone reaction. Then, the parental DNA was DpnI digested and the remaining pET16bP construct harboring the mutation was transformed into *E. coli* BL21 cells for gene expression. Successful mutation of the target genes was proven by sequencing of the plasmid using the pET16-check-fw/pET16-check-rev primer [34].

3.5. Protein purification and quantification

Recombinant protein production and purification was done as described previously [9,23].

Protein concentration was determined by means of the Bradford method employing bovine serum albumin as a standard [35]. The purity of protein preparations was controlled by SDS-PAGE as described earlier for those types of proteins [5,6].

Further, the FAD content of proteins was determined by the protocol employed for *Ro*StyA1 and *Ro*StyA2B [6]. Therefore, authentic standards for FMN and FAD had been determined by RP-HPLC connected to a diode array detector for UV/Vis range.

3.6. Enzyme assays and product analysis

Flavin oxidoreductase activity was determined spectrophotometrically (Cary 50, Varian) by quantifying NAD(P)H consumption at 340 nm (ε_{340} nm = 6.22 mM^{-1} cm^{-1}). One unit of enzyme activity is defined as the amount required to oxidize 1 µmol of NAD(P)H per min. All measurements were carried out in triplicate. The standard assay (1 mL) consisted of 20 mM Tris-HCl, pH 7.5, 68 µM flavin co-substrate (FAD, FMN or riboflavin) and 177 µM NADH. After incubating the mixture for 10 min at 30 °C, the reaction was started by adding an appropriate amount of enzyme. For estimating steady-state kinetic parameters, initial reaction rates were determined using 4.1 to 90.2 µM FAD and 7.9 to 164 µM NADH. Kinetic parameters were obtained by nonlinear regression analysis applying KaleidaGraph 4.5 (Synergy Software), assuming Michaelis-Menten kinetics.

Monooxygenase activity assay and standard HPLC analytic were performed as described previously [5,9]. Reduced FAD was supplied by an additional reductase *Ro*StyBart [23] or 10 mM BNAH, respectively.

The 2h biotransformations were done according to the standard monooxygenase assay by adding the respective sulfides instead of styrene as substrate. Enantiomeric excess of obtained products were analyzed as described previously for epoxides [5] and sulfoxides [26].

4. Conclusions

The styrene monooxygeanse *Vp*StyA1 and *Vp*StyA2B originating of *Variovorax paradoxus* EPS was herein described as the first proteobacterial E2 type representative which also employs a fusion protein as an NADH:FAD-oxidoreductase. Both proteins were successfully produced by recombinant expression. The enzyme converts styrene in an enantioselective manner as well as a number of aryl alkyl sulfides. However, it was by far more active (3.3 to 22-times) on these sulfides as on styrene. Therefore, this enzyme can be proposed as an enantioselective sulfoxidase. Furthermore, the potential fusion region of StyA2B-like proteins was identified and mutated. It was demonstrated that this region has effects on the reductase as well as monooxygenase domain, and it is especially critical for the FAD binding, probably of both domains. Structural and kinetic studies may reveal the effect of the fusion on the individual domains of *Vp*StyA2B and related monooxygenase. Thus, this SMO of strain EPS is a suitable candidate for structural investigations as well as to develop an enantioselective catalyst for sulfoxidation reactions.

Supplementary Materials: The following are available online at www.mdpi.com/link, Figure S1: Sensitivity of *Vp*StyA2B towards putative inhibitors determined by applying the NADH:FAD oxidoreductase assay, Table S1: Strains, plasmids and primers used in this study.

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Sample Availability: Samples of the compounds or plasmids are available from the authors.