Catalytic and hydrodynamic properties of styrene monooxygenases from *Rhodococcus opacus* 1CP are modulated by cofactor binding

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**Abstract**

Styrene monooxygenases (SMOs) are flavoenzymes catalyzing the epoxidation of styrene into styrene oxide. SMOs are composed of a monooxygenase (StyA) and a reductase (StyB). The latter delivers reduced FAD to StyA on the expense of NADH. We identified *Rhodococcus opacus* 1CP as the first microorganism to possess three different StyA isoforms occurring in two systems StyA1/StyA2B and StyA/StyB, respectively. The hydrodynamic properties of StyA isozymes were found to be modulated by the binding of the (reduced) FAD cofactor. StyA1 and SyA2B mainly occur as dimers in their active forms while StyA is a monomer. StyA1 showed the highest epoxidation activity and excellent enantioselectivity in aromatic sulfoxidation. The hydrodynamic and biocatalytic properties of SMOs from strain 1CP are of relevance for investigation of possible industrial applications.

**Keywords:** Flavoprotein, Monooxygenase, Oligomerization, FAD binding, *Rhodococcus opacus* 1CP, Styrene epoxidation

**Introduction**

Styrene monooxygenases (SMO; EC 1.14.14.11) are two-component flavoenzymes composed of a monooxygenase (StyA) and a FAD reductase (StyB) (Hartmans et al. 1990; Huijbers et al. 2014; Montersino et al. 2011; van Berkel et al. 2006). StyB releases electrons from NADH to oxidized FAD which is then translocated in its reduced state to StyA. Incorporation of reduced FAD in StyA and subsequent reaction with molecular oxygen yields flavin hydroperoxide, which stimulates the binding of styrene and its subsequent epoxidation (Kantz and Gassner 2011).

SMOs have been extensively characterized with respect to the enantioselective conversion of styrene derivatives (Panke et al. 2000; Park et al. 2006a, b; Tischler et al. 2009). The first SMOs studied originated from bacterial isolates of various soil samples (Hartmans et al. 1990), followed by SMOs from *Pseudomonas fluorescens* ST (Beltrametti et al. 1997; Gennaro et al. 1999; Marconi et al. 1996), *Pseudomonas* spp. Y2 (Velasco et al. 1998) and VLB120 (Hollmann et al. 2003; Otto et al. 2004), *Pseudomonas putida* S12 (Kantz et al. 2005; Morrison et al. 2013; Ukaegbu et al. 2010), *Pseudomonas putida* CA-3 (Nikodinovic-Runic et al. 2013; O’Conner et al. 1995), metagenome screening (van Hellemont et al. 2007), and *Rhodococcus* spp. ST-5 and ST-10 (Toda et al. 2012; Toda and Itoh 2012).

The first one-component SMO (StyA2B) was discovered from *Rhodococcus opacus* 1CP representing a natural fusion between the oxygenase and reductase subunits (Tischler et al. 2009). The epoxidase activity of StyA2B appeared to be rather low. However, a second oxygenase (StyA1), present in the same gene cluster, boosted the epoxidase activity when StyA2B was used as partner reductase (Tischler et al. 2010). More recently, we found another SMO cluster in the *R. opacus* 1CP genome (KF540256) (Oelschlägel et al. 2014). This *styABCD* cluster is similar to those of pseudomonads and *Rhodococcus*...
sp. ST-5. Remarkably, this discovery makes *R. opacus* 1CP the first organism possessing both one- and two-component SMOs (Figures 1, 2) raising the question about the evolution of the two types of SMOs.

SMO oxygenase and reductase components usually occur as homodimers (Morrison et al. 2013; Otto et al. 2004; Tischler et al. 2010; Ukaegbu et al. 2010). StyAs from *Rhodococcus* sp. ST-5 and ST-10 were recently reported to be in monomer–dimer equilibrium (Toda et al. 2012). However, these proteins were studied in their apo-form and the effect of flavin binding was not considered. Because oligomerization might influence the catalytic efficiency of SMO systems (Otto et al. 2004), we here address the hydrodynamic properties of StyA1, StyA2B and StyA from *R. opacus* 1CP in the absence and presence of oxidized or reduced FAD. The results obtained are discussed in relation to the catalytic and structural features of SMOs.

**Materials and methods**

**Chemicals**

Styrene, styrene oxide, phenyl vinyl sulfide, phenyl vinyl sulfoxide, and cofactors were purchased from Sigma-Aldrich (Steinheim, Germany) and Carl Roth (Karlsruhe, Germany). Restriction enzymes were received from MBI Fermentas (St. Leon-Rot, Germany) and New England Biolabs GmbH (Frankfurt am Main, Germany). Oligonucleotides and synthetic genes were synthesized by Eurofins MWG Operon (Ebersberg, Germany).

**Bacterial strains, plasmids, and culture conditions**

Bacterial strains and plasmids used in this study were treated according to Tischler et al. (2009). Other plasmids and primers are listed in Table 1. *Escherichia coli* BL21 strains were grown aerobically in LB-media (100 µg mL⁻¹ ampicillin, and 50 µg mL⁻¹ chloramphenicol) at 37°C while shaking constantly at 120 rpm in baffled flasks.

The strains *Rhodococcus opacus* 1CP (DSMZ; DSM 46757, and VKM; Ac-2638) and *Pseudomonas fluorescens* ST (DSMZ; DSM 6290) are available from public culture collections.

**Construction of expression clones**

The *styA* gene was amplified from genomic *R. opacus* 1CP DNA by PCR (annealing temperature 58.6°C) by

![Figure 1](image-url)
applying the appropriate primers (Table 1). The purified products were cloned into pET1.2/blunt cloning vector using the CloneJET PCR Cloning Kit (Thermo Scientific).

The styB gene originating from strain 1CP was codon optimized (accession number: KP711388) and cloned in a pEX-A vector system flanked by the restriction sites NdeI and NotI. The GC content of styB was adapted to the codon usage of strain Acinetobacter sp. ADP1 allowing for higher gene expression levels (with E. coli BL21 or...
alternatively with Acinetobacter species as host) yielding soluble protein. The received vector was used in analogy to the styA cloning procedure to yield a styA construct of pET16bp designated as pSRoB_P01.

Another styB gene was amplified from Pseudomonas fluorescens ST (Beltrami et al. 1997) by PCR (annealing temperature $\Delta T = 54.8^\circ C$) by applying the appropriate primers (Table 1). Products obtained were cloned into pJET1.2/blunt cloning vector as described above. To yield the expression construct pSPB_P01, styB was digested (KpnI) and subsequently ligated into similarly treated pET16bp.

**Gene expression, protein purification and storage**

Recombinant proteins were obtained as His$_{10}$-tagged fusion proteins. The styA, styA1 and styA2B expression took place in a 5-L biofermentor as described previously (Tischler et al. 2009, 2010), or in 2-L flasks, respectively. When cell density reached an OD$_{600}$ of 0.5, induction was started by addition of a 0.05 mM IPTG (isopropyl-$\beta$-D-thiogalactopyranoside) (120 rpm, 22 h, 20°C). Cells were harvested by centrifugation (5,000 $\times$ g, 30 min, 4°C), resuspended in 10 mM Tris–HCl (pH 7.5), and $\beta$-mercaptoethanol and excess salt, proteins were treated with a 10 mL Amicon Ultra Centrifugal Filters in the presence of 10 mM Tris–HCl (pH 7.5), 175 µM NADH, 60 µM FAD, and an appropriate amount of recombinant StyA2B or StyB, respectively. After 10 min of pre-incubation at 30°C, the reaction was started by adding NADH. Checkpoints were taken at 2 s interval. Initial reaction rates were measured using 2–300 µM FAD or NADH while keeping the corresponding co-substrate at a constant concentration in excess. In case of StyB from strain 1CP higher concentrations of substrates were necessary and therefore the NADH consumption was determined at 320 nm ($e = 4.65 \text{mM}^{-1} \text{cm}^{-1}$).

Monoxygenase activity of recombinant StyA, StyA1, and StyA2B with styrene or phenyl vinyl sulfide was determined via quantification of the products styrene oxide or phenyl vinyl sulfoxide, respectively. The enzymatic assay and standard HPLC analysis were performed as described previously (Tischler et al. 2009). The protocol was modified in order to determine the sulfide and sulfoxide via HPLC. Isocratic elution of respective compounds occurred with a 40% methanol/water eluent at a flow rate of 0.7 mL min$^{-1}$. Products obtained were analyzed for enantiomers formed according to previously performed methods for epoxides (Tischler et al. 2009) or for sulfoxides (Anderson et al. 2002).

Protein content was determined using BCA Protein Assay Reagent (Thermo Scientific) or with the Bradford method using Protein Assay Reagent (Bio-Rad), respectively. Bovine serum albumin (Sigma) was used as a standard. Purity of protein batches was controlled by SDS-PAGE analysis (see Additional file 1). A purity of 95% or higher was found for all proteins even of various expression attempts of same protein. The purity of these batches was considered in calculating the activity values and for analytical gel filtration.

**Analytical gel filtration**

The hydrodynamic properties of SMOs were analyzed on an Äktaexplorer FPLC system (Pharmacia Biotech) applying a Superdex 200 HR 10/30 column (bed volume $V_i = 22.0 \text{mL}$, GE Healthcare Life Sciences). The flow rate was 0.6 mL min$^{-1}$ and the temperature was kept at 22°C. 100 µL of sample solution (~2 mg mL$^{-1}$ protein of 95% or higher purity) was separated by applying a
mobile phase containing 10 mM Tris–HCl (pH 7.2), and 500 mM sodium chloride. The elution behavior of oxidized holoenzymes was studied by adding 12.7 µM FAD to both buffer and sample solution. For studying the elution behavior of the reduced holoenzymes, 1 mM sodium dithionite was applied to all FAD-containing solutions. For the latter the FPLC system was extensively washed with anaerobic buffer containing 1 mM sodium dithionite. The identity of the eluted proteins was checked by analysis of collected fractions on SDS-PAGE.

The apparent molecular masses ($M_r$) of SMOs were determined from running the following calibration proteins under similar conditions: myoglobin (17.8 kDa, 17.3 mL), chymotrypsin (25 kDa, 17.1 mL), ovalbumin (42.8 kDa, 15.3 mL) and bovine serum albumin (68 kDa, 14.3 mL and 136 kDa, 12.5 mL). Dextran blue (2,000 kDa) was used to determine the void volume ($V_0 = 7.9$ mL). Apparent $M_r$ values of SMOs were obtained from a graph where the partition coefficients ($K_{av}$) of the standard proteins were plotted against log $M_r$: 

$$M_r = 10^{\frac{\log_{10} K_{av} - 1.1884}{-0.4035}}.$$

**Results**

**Identification of a novel two-component styrene monoxygenase from *Rhodococcus opacus* 1CP**

The discovery of the *styA2B* gene in a cluster with *styA1* (Tischler et al. 2009) prompted the search of other styrene catabolic genes in *R. opacus* 1CP. Interestingly, a third SMO gene *styA* was found within a *styABCD* cluster (accession number: KF540256) (Figure 1) of the recently completed genome sequence of *R. opacus* 1CP (Oelschlägel et al. 2014). Next to the *styA* gene, the *styABCD* cluster harbors genes for a flavin reductase (*styB*), as well as styrene oxide isomerase (*styC*) and phenylacetaldehyde dehydrogenase (*styD*). A highly related gene cluster is present in *Rhodococcus* sp. ST-5 (Figure 1) (Toda and Itoh 2012). Further, similar chromosomal regions have been described for *Pseudomonas fluorescens* ST (Beltrametti et al. 1997), *Pseudomonas putida* SN1 (Park et al. 2006a, b), *Pseudomonas* sp. Y2 (Velasco et al. 1998), as well as *Pseudomonas* sp. VLB120 (Panke et al. 1998).

The *styA* and *styB* genes from *R. opacus* 1CP encode for proteins with 427 and 178 amino acid residues with calculated molecular masses of 46,580 and 19,053 Da, respectively. The deduced amino acid sequence of *StyA* showed high identity to *StyA* from *Rhodococcus* sp. ST-5 (81%), and ST-10 (73%), as well as to *StyA* proteins from pseudomonads (~59%). However, the identity to *StyA1* and the oxygenase part of *StyA2B* (aa 1–413) is only 28–29% in both cases.

The deduced amino acid sequence of flavin reductase *StyB* showed most identity with *StyB* from *Rhodococcus* sp. ST-5 (81%) and ST-10 (62%), and moderate identity with *StyB* proteins from pseudomonads (44–47%). The identity to the reductase part of *StyA2B* (aa 414–573; 160 aa) is rather low (25%).

In summary, a novel two-component styrene monoxygenase was identified in *R. opacus* 1CP that shares high sequence identity and gene cluster organization to the corresponding systems from *Rhodococcus* sp. ST-5 and to those of pseudomonads (Figure 1).

**Overexpression and purification of *StyA* and *StyB**

The *styA* and *styB* genes from *R. opacus* 1CP were successfully cloned into pET16bp and transformed into *E. coli* BL21. When expression was successful, a blue staining could be observed due to formation of indigo from indole originating from tryptophan. From *StyA* expression, a cell dry weight of 1.2 g and 7.8 mg of soluble *StyA* protein per liter culture was obtained.

*StyB* was produced by expression of the BL21 cells in high salt LB-media (0.5 M NaCl) containing extra 2.5 mM betaine and 0.2% glucose. From *styB* expression, a cell dry weight of 1.4 g and 17.4 mg soluble *StyB* protein per liter culture was obtained.

Recombinant His$_{10}$-tagged *StyA* and *StyB* proteins were purified via immobilized metal ion affinity chromatography (IMAC). Collected fractions were analyzed by SDS-PAGE showing that *StyA* as well as *StyB* are highly pure after the described procedure (see Additional file 1). Recombinant *StyA* possesses an apparent subunit molecular mass of 50 kDa including the His-tag while *StyB* exhibits an apparent subunit molecular mass of 21.5 kDa, in agreement with the deduced gene sequences.

*StyA1* and *StyA2B* were obtained from expression attempts and protein purification as described earlier (Tischler et al. 2009, 2010). Purity was controlled by SDS-PAGE prior further analysis and application in biotransformation.

*StyB* from *R. opacus* 1CP was obtained in soluble form, which is rather unusual for *StyB*-homologs (Otto et al. 2004; Yeo et al. 2009). Maximum activities of 75.1 ± 1.8 and 91.9 ± 6.6 U mg$^{-1}$, and $K_M$ values of 59.3 ± 4.2 and 108.3 ± 18.3 µM were determined for the substrates NADH and FAD, respectively (Figure 2; Table 2).

*StyB* from *R. opacus* 1CP was found to be unstable in time and not suitable for SMO epoxidation experiments. For that purpose (vide infra), we used *StyB* from *P. fluorescens* ST. *StyB* from strain ST was successfully refolded from inclusion bodies to give a highly pure and active FAD reductase (Table 2). *StyB* from strain ST showed maximum activities of 28.8 ± 0.4 and of 28.7 ± 1.0 U mg$^{-1}$, and $K_M$-values of 18.9 ± 1.0 and of
2.6 ± 0.4 µM for the substrates NADH and FAD, respectively. The catalytic efficiency of StyB from strain ST is comparable to that of StyB from strain 1CP, mainly because of a better affinity for both NADH and FAD (Table 2).

**StyA1 is the most active styrene monooxygenase from strain 1CP**

Oxygenase activities of StyA1, StyA2B and StyA were determined by measuring the product of styrene oxidation (styrene oxide) via HPLC. StyB reductase, formate dehydrogenase and catalase were added in order to produce reduced FAD, regenerate NADH, and remove hydrogen peroxide. All StyA enzymes were incubated with a threefold excess of StyB from *P. fluorescens* ST over StyAs.

From these experiments StyA1 showed a specific styrene epoxidation activity of 0.12 ± 0.02 U mg⁻¹ while StyA and the fusion protein StyA2B revealed specific activities of 0.08 ± 0.01 and 0.037 ± 0.01 U mg⁻¹, respectively. StyAs from *Rhodococcus* ST-5 (0.03 U mg⁻¹) and ST-10 (0.026 U mg⁻¹) were reported to possess lower activities (Toda et al. 2012). These findings state StyA1 and StyA from *R. opacus* 1CP as the most active SMOs from rhodococci so far.

Product analysis confirmed an earlier finding (Tischler et al. 2010) that StyA1 and StyA2B are able to epoxidize styrene to the (S)-enantiomer with an ee of 94%. The newly discovered StyA catalyzed the formation of this enantiomer with an ee exceeding 97% (Figure 3).

Besides epoxidation also the sulfoxidation was assayed. The substrate chosen was phenyl vinyl sulfide in order to provide a sulfoxidation as well as an epoxidation site (Figure 3). All three SMO isoforms were found to convert this substrate and only sulfoxidation was determined. The activities are comparable to styrene epoxidation. Notably, StyA2B showed a 2.4-times higher sulfoxidase as epoxidase activity. All three SMO isoforms were found to catalyze the sulfoxidation in an enantioselective manner. However, StyA1 stood out by producing more than 99% S-enantiomer of phenyl vinyl sulfoxide.

**Quaternary structure of SMO systems**

Figure 4 shows the elution behavior of apo-, holo-, and reconstituted StyA1 on a Superdex200 gel filtration column. For the apoenzyme the main fraction eluted around 14.3 mL, while smaller fractions eluted around 13.0, 11.1 and 8.2 mL. This suggests that under the conditions applied, apo-StyA1 mainly occurs as a monomer, in equilibrium with dimers and higher-order quaternary forms. For holo-StyA1, the equilibrium shifted towards the dimeric form. When holo-StyA1 was fully reduced, the enzyme was almost exclusively present as a dimer (13.8 mL) and only a small fraction of oligomers remained.

StyA2B formed dimers as well as larger species (Figure 4). For both apo- and holo-StyA2B considerable amounts of higher-order quaternary forms were observed. Under reduced conditions, a clear shift into the direction of dimers (12.9 mL) occurred.

A strikingly different hydrodynamic behavior was observed for StyA (Figure 4). StyA mainly was found as a monomer (15.2 mL) under the conditions applied. In the apo-state approximately 10% tetramer (11.5 mL) and 30% dimer (13.7 mL) was found next to the monomer. When FAD was added, the amount of monomer increased to 80%, while the tetramer no longer existed. In the reduced form, holo-StyA was almost completely present as a monomer. The elution volume of the monomer decreased upon binding FADred and FADox resulting in an apparent molecular mass shift of about 2–4 kDa. This shift arises probably due to the stronger binding of reduced FAD shifting the fast monomer–dimer equilibrium present in the apo form towards the pure monomer form resulting in a somewhat larger elution volume.

StyB from *Rhodococcus opacus* 1CP was found to occur as dimer in the presence of the cofactor FAD (not shown). Due to its low stability, no data were collected for the holoprotein under reduced conditions.

**Discussion**

We found that the Gram-positive actinobacterium *Rhodococcus opacus* 1CP contains three different SMOs.
Next to the previously reported styA1 and styA2B genes (Tischler et al. 2009), the third SMO gene is located within a styABCD cluster, completely separate from styA1 and styA2B. The styABCD cluster of R. opacus 1CP differs from styrene clusters of Pseudomonas species (Figure 1). The latter clusters exhibit a pathway-specific regulatory apparatus of two sensor kinases StyS and StyR as well as a transcriptional repressor PaaX (Panke et al. 1998; Velasco et al. 1998; Yeo et al. 2009). Similar sensor kinases are lacking in R. opacus 1CP as well as in Rhodococcus sp. ST-5 (Toda and Itoh 2012), but a PaaX-like regulator is present upstream from styA1/styA2B in strain 1CP (Tischler et al. 2009). Thus, in strain 1CP, both the styA1/styA2B gene cluster and the newly discovered styABCD-cluster might be regulated differently compared to that found for Pseudomonas species (Alonso et al. 2003; Oelschlägel et al. 2014). The presence of three StyA isoenzymes in a single strain is new and underlines the gene redundancy of rhodococci (Gröning et al. 2014a; Patrauchan et al. 2005). The different gene cluster organization as well as the respective regulatory machinery might indicate a convergent evolution (Tischler et al. 2012).

The newly discovered FAD reductase StyB from strain 1CP was found to be highly active with NADH as electron donor. However, in comparison to StyB-homologs (Otto et al. 2004; Tischler et al. 2009; Toda et al. 2012), the binding affinity towards NADH and FAD is rather low. StyB forms a homodimer, as reported for other StyB enzymes (Gröning et al. 2014b; Otto et al. 2004; Toda et al. 2012), but appeared to be rather unstable, limiting its further characterization.

StyA enzymes become active after receiving a reduced FAD molecule from their StyB partner. The tightly bound reduced flavin then reacts with molecular oxygen yielding a flavin hydroperoxide that acts as oxygen donor in the subsequent epoxidation reaction (Kantz and Gassner 2011). Here, by using StyB from

| SMO                  | Expression rate** [mg L⁻¹] | Substrate: styrene Specific activity [U mg⁻¹] | Enantiomeric excess [%] | Substrate: phenyl vinyl sulfide Relative activity [%] | Enantiomeric excess [%] |
|----------------------|----------------------------|-----------------------------------------------|-------------------------|-----------------------------------------------|-------------------------|
| StyA + StyB*         | 7.80                       | 0.081                                         | > 97 (S)                | 28.6                                          | > 79 (S)                |
| StyA1 + StyB*        | 1.86⁺                      | 0.121                                         | > 96 (S)⁺               | 56.7                                          | > 99 (S)                |
| StyA2B + StyB*       | 8.00ᵇ                      | 0.037                                         | > 94 (S)ᵇ               | 239.2                                         | > 71 (S)                |

ND not determined. * StyB originated from Pseudomonas fluorescens ST and in case of StyA2B the additional reductase activity increased the monoxygenase activity of the fusion protein. ** Expression rate was determined from StyA expression. ³Tischler et al. 2010; bTischler et al. 2009. *** Sulfoxide produced according to a reference assay with styrene as substrate (100% represents the yield of styrene oxide after 2 h biotransformation).

Figure 3 Specific activities and enantioselectivities of SMOs from R. opacus 1CP
*P. fluorescens* ST as partner reductase, we show that the oxygenation activity of StyA is in between that of StyA1 and StyA2B (Figure 3) and higher than that of StyA enzymes from other rhodococci. The StyA isoforms convert phenyl vinyl sulfide specifically into the corresponding sulfoxide with a similar activity as with styrene. Especially StyA1 shows excellent enantioselectivity in the sulfoxidation reaction by producing more than 99% of the S-enantiomer. With all three StyA isoforms no over-oxidation and no epoxidation of the adjacent vinyl chain was observed. This is important for biocatalytic applications and indicates that the vinyl moiety of phenyl vinyl sulfide is bound in the StyA active sites remote from the flavin hydroperoxide.

The successful combination of StyA enzymes from *R. opacus* 1CP with a foreign FAD reductase supports a diffusible transfer mechanism of reduced FAD (Hollmann et al. 2003; Kantz et al. 2005; Morrison et al. 2013). Here, we obtained evidence that such a mechanism can occur with both monomeric and dimeric StyA forms. After purification, the StyA isoforms occurred in their apo-form. Analytical gel filtration revealed that the

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**Figure 4** Size exclusion chromatography on Superdex-200 of apo-, holo- and reconstituted StyAs. Left: Samples of 100 µL protein (1.95 mg mL$^{-1}$ StyA1, 2.17 mg mL$^{-1}$ StyA2B, 11.45 mg mL$^{-1}$ StyA) were loaded onto the column. Elution was performed in 10 mM Tris–HCl (pH 7.2), 500 mM sodium chloride, pH 7.2 at 22°C. The flow rate was 0.6 mL min$^{-1}$. Reconstituted enzymes were prepared by incubating both protein and buffer with 12.7 µM FAD, either in the absence or presence of 1 mM sodium dithionite. Right: Distribution of the isoforms in the three conditions: apo, holo and reduced StyA. Intensity of arrows shows dominant direction of equilibrium (compare with Table 3).
hydrodynamic properties of the isoforms change in the presence of (reduced) FAD. StyA1 and StyA2B constitute dimers under reduced conditions while StyA is a monomer. This shows for the first time that StyA oxygenases can be active as monomers.

Our data clearly indicate that StyA1 is a dimer in its active form. This raises the question about how the dimer interface of this enzyme is formed. StyA1 might form a ‘back to back’ dimerization interface, which resembles that of related monooxygenases with a para-hydroxybenzoate hydroxylase (PHBH) fold (Montersino et al. 2013; Schreuder et al. 1988). Such mode of interaction seems advantageous for the binding of substrates and efficient catalysis, since in such a dimer the active sites point into the surrounding medium and provide space for the entering substrates. Especially, when reduced FAD is transferred from the reductase to the oxygenase component this might be beneficial to avoid auto-oxidation of FAD (Morrison et al. 2013). Interestingly, in the crystal structure of apo StyA from Pseudomonas sp. S12, the active sites of the dimer are located face to face (Figure 5a) (Ukaegbu et al. 2010). This orientation might not necessarily represent the active form since no data are available for the reduced protein. Thus, the structure of StyA1 with reduced FAD bound would be favorable to understand better the FAD transfer mechanism.

In StyA2B the StyB reductase is fused to the StyA2 oxygenase. Most StyB reductases occur as dimers (Gröning et al. 2014b; Morrison et al. 2013; Otto et al. 2004; van den Heuvel et al. 2004), and we also observe a dimeric nature for StyA2B and StyB from strain 1CP. From the apparent molecular mass determined by gel filtration ($M_r = 111 \pm 5$ kDa; Figure 3; Table 3) we conclude that StyA2B has a globular fold. This strongly suggests that in one StyA2B subunit, two dimer interfaces (epoxidase and reductase domain) are present (Figure 5c, d) and that StyA2 forms a ‘double-back to back’ dimer.

The reason why the hydrodynamic state of investigated SMOs varies between the resting state and the active state is not simple to answer. Since we are dealing with isolated proteins, it could be that the heterologous produced StyA enzymes have a tendency to aggregate in the absence of their cofactor. On the other hand, the differences in oligomerization observed might point to an important regulatory mechanism, which still needs to be uncovered. It should be noted here that the possible interaction with the gel filtration material as well as the shape of the SMO proteins were not considered in this study. Additional studies from analytical ultracentrifugation or matrix-assisted light scattering might help to enlighten the aggregation behavior of the SMO proteins in further detail.

**Conclusion**

In conclusion, the differences in hydrodynamic properties of StyA monooxygenases from strain 1CP depict additional evidence for a convergent evolution of these enzymes. Binding of (reduced) FAD to StyA enzymes inhibits their aggregation and results in either monomeric (StyA) or dimeric (StyA1, StyA2B) active forms. The present results suggest that incorporation of reduced FAD into StyA enzymes is attended with significant conformational rearrangements. Changes in protein–flavin interaction might also occur during catalysis, as previously observed for other monooxygenases with a PHBH fold (Huijbers et al. 2014; Montersino et al. 2011, 2013; Schreuder et al. 1988). More insight into the binding mode of reduced FAD and aromatic

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**Figure 5** Schematic view on possible dimer structures of styrene monooxygenase components. StyA has the same fold as PHBH and the domains were accordingly colored (Montersino et al. 2011, 2013; Schreuder et al. 1988). FAD binding domain in green, substrate binding domain in red, and dimer interface in blue. The three-dimensional structure of StyA from Pseudomonas sp. S12 (PDB ID: 3IHM, Ukaegbu et al. 2010) is shown in (a), where the active site clefts of monomers point to the center and no PHBH-like dimer was determined. A modelled StyA-dimer is presented in (b). Here the dimer interfaces interact in a flavoprotein hydroxylase mode (Montersino et al. 2013; Schreuder et al. 1988) and a ‘back to back’ situation is shown in which active sites point into the medium. The experimentally solved three-dimensional StyB-structure (PDB ID: 4F07, Morrison et al. 2013) is shown as dimer in (c). Together the structures of (a) and (c) were used to generate a model of StyA2B (d) in which two dimer interfaces occur (van den Heuvel et al. 2004). For clarity, only one StyA2B monomer is shown.
substrates is of utmost importance for understanding the catalytic potential and enantioselectivity of styrene monoxygenases.

### Additional files

**Additional file 1:** In the Supplemental Material Section results from the protein purification and respective SDS-PAGE as well as the data from calibration runs for the analytical gel filtration are presented.

### Authors’ contributions

AR, TH and CC carried out the molecular genetic studies and recombinant protein production. DT, AR, TH and AHW carried out the analytical gel filtration and kinetics (data acquisition and analysis). Product analysis was established and carried out by PR. AR, DT and WJHB drafted the manuscript which was critically revisited by all authors. All authors read and approved the final manuscript.

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### Compliance with ethical guidelines

### Competing interests

The authors declare that they have no competing interests.

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### Table 3 Hydrodynamic properties of StyA oxygenases from *R. opacus* 1CP as monitored by analytical gel filtration in the absence or presence of (reduced) FAD

| StyA oxygenase | Elution volume (mL)a | Apparent molecular mass (kDa)a | Hydrodynamic state |
|----------------|----------------------|--------------------------------|-------------------|
| StyA1          | 14.3                 | 66                             | Monomer–Dimer     |
|                | 14.0                 | 75                             | Monomer–Dimer     |
|                | 11.1                 | 241                            | Oligomer          |
|                | 8.2                  | >                              | Polymer           |
| StyA1 + FADox | 14.3                 | 66                             | Monomer–Dimer     |
|                | 14.0                 | 74                             | Monomer–Dimer     |
|                | 10.8                 | 273                            | Oligomer          |
|                | 7.9                  | >                              | Polymer           |
| StyA1 + FADred| 13.8                 | 81                             | Dimer             |
| StyA2B         | 12.9                 | 116                            | Dimer             |
|                | 9.4                  | >                              | Oligomer          |
|                | 8.3                  | >                              | Polymer           |
| StyA2B + FADox| 13.0                 | 112                            | Dimer             |
|                | 9.4                  | >                              | Oligomer          |
|                | 8.3                  | >                              | Polymer           |
| StyA2B + FADred| 13.1                | 107                            | Dimer             |
| StyA           | 15.2                 | 46                             | Monomer           |
|                | 13.7                 | 84                             | Dimer             |
|                | 11.5                 | 205                            | Tetramer          |
| StyA + FADox  | 15.1                 | 48                             | Monomer           |
|                | 13.5                 | 91                             | Dimer             |
| StyA + FADred | 15.0                 | 50                             | Monomer           |

*a* Superdex 200 column, bed volume = 22.0 mL. The mean of apparent molecular mass of repeated runs of protein samples is shown and the calculated standard deviation was between 5 and 10% in all cases.
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