StyA1 and StyA2B from *Rhodococcus opacus* 1CP: a Multifunctional Styrene Monoxygenase System

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Two-component flavoprotein monoxygenases are emerging biocatalysts that generally consist of a monoxygenase and a reductase component. Here we show that *Rhodococcus opacus* 1CP encodes a multifunctional enantioselective flavoprotein monoxygenase system composed of a single styrene monoxygenase (SMO) (StyA1) and another styrene monoxygenase fused to an NADH-flavin oxidoreductase (StyA2B). StyA1 and StyA2B convert styrene and chemical analogues to the corresponding epoxides at the expense of FADH2 provided from StyA2B. The StyA1/StyA2B system presents the highest monoxygenase activity in an equimolar ratio of StyA1 and StyA2B, indicating (transient) protein complex formation. StyA1 is also active when FADH2 is supplied from StyB of *Pseudomonas* sp. VL8120 or PheA2 from *Rhodococcus opacus* 1CP. However, in both cases the reductase produces an excess of FADH2, resulting in a high waste of NADH. The epoxidation rate of StyA1 heavily depends on the type of reductase. This supports that the FADH2-induced activation of StyA1 requires interprotein communication. We conclude that the StyA1/StyA2B system represents a novel type of multifunctional flavoprotein monoxygenase. Its unique mechanism of cofactor utilization provides new opportunities for biotechnological applications and is highly relevant from a structural and evolutionary point of view.

The environmentally harmful hydrocarbon styrene is readily biodegradable by various classes of microorganisms covering Gram-negative and Gram-positive bacteria as well as fungi (e. g., ascomycetes). Two major pathways for styrene mineralization have been described (reviewed in references 23, 26, and 33); of these, the most common one is initiated by a monoxygenase-catalyzed epoxidation of the vinyl side chain. Due to their biotechnological potential, the styrene monoxygenases (SMOs) involved in this reaction have received considerable attention. Most SMOs have been described for pseudomonads and were investigated for their biochemical properties (5, 13, 27, 31, 46) and their biotechnological applicability in cell-free (16, 17) or whole-cell systems (3, 12, 28, 29, 30, 32, 37). All SMOs investigated thus far convert styrene in a highly enantioselective manner to (S)-styrene oxide, which is a useful precursor for several chiral synthons and pharmaceuticals (2, 6, 14, 26, 34). Moreover, the relaxed substrate specificity of SMOs allows an enantioselective conversion of substituted styrene derivatives and structurally related compounds, like indene and dihydronaphthalene, as well as phenylalkylsulfides (Fig. 1) (17, 40, 45), thus increasing their biocatalytic potential.

Typical SMOs of pseudomonads consist of two enzymatically active protein components encoded by genes that are usually clustered adjacent to each other (styA and styB) (Fig. 2a) (26, 43, 46). The flavin reductase subunit (StyB) reduces flavin adenine dinucleotide (FAD) at the expense of NADH.

The monoxygenase subunit (StyA) then utilizes the reduced flavin (FADH2) to activate molecular oxygen for styrene attack (Fig. 2b). The mechanism of FAD transfer between StyB and StyA is not yet fully understood. In vitro, StyB can be replaced by different flavin reductases from other organisms (e.g., oxidoreductases from *Photobacterium fischeri* or *Geobacillus thermoglucosidasius*) (27, 45), or FADH2 can be supplied electrochemically (16, 17). In both cases, StyA shows no significant loss in epoxidation capacity, indicating that no specific protein-protein interactions are needed for FADH2 transfer. Indications for a protein contact-based transfer of FADH2 were found by kinetic studies (19) and have also been proposed for other two-component flavoprotein monoxygenases (9, 10). Efficient interprotein transfer of the flavin cofactor will prevent FADH2 autooxidation and limit oxidative stress (21, 22). Preventing such stress is highly relevant for the biotechnological applicability of multicomponent oxygenases since it can severely improve long-term stability of whole-cell as well as cell-free transformation processes.

An evolutionary strategy to overcome transport-based limitations is the generation of self-sufficient fusion proteins (24, 35, 38). The hemoflavoprotein P450 BM3 from *Bacillus megaterium* is a well-known prototype, and its high oxygenation efficiency has triggered activities of protein engineering in order to construct novel protein chimeras by gene fusion (24, 25, 41). Recently, we reported on the identification of the first self-sufficient styrene monoxygenase from the nocardioform actinobacterium *Rhodococcus opacus* 1CP (40). This novel enzyme (StyA2B) harbors a monoxygenase and reductase unit in one polypeptide chain. The recombinant protein was biochemically characterized showing similar substrate specificity and enantioselectivity levels compared to those of two-component SMOs. However, the specific activity of the StyA2B

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a typical two-component SMO of Pseudomonas hypothetical cooperation between StyA2B and StyA1. The FADH2 surplus of StyA2B is utilized by StyA1, yielding an increase of styrene.

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Based on the above-mentioned notion of a high-performance monooxygenase. A closer look at the genetic localization of styA2B indicated the presence of an open reading frame (styA1) encoding another putative styrene monooxygenase (Fig. 2a). Interestingly, a similar open reading frame is present directly upstream of other fused SMO genes in the genomes of Nocardia farcinica IFM10152 and Arthrobacter aurescens TC1 (40, 45). This structural conservation and feasibility of cotranscription may indicate a functional dependency (Fig. 2b). To address this issue, we studied the catalytic properties of the StyA1/StyA2B system. Our data indicate that this styrene-oxygenating system represents an unprecedented type of two-component flavoprotein monooxygenase.

MATERIALS AND METHODS

Chemicals and enzymes. Oxygenation substrates, (S)- and (R)-styrene oxide, catalase, formate dehydrogenase, and cofactors were purchased from Sigma-Aldrich (Steinheim, Germany) and Carl Roth (Karlsruhe, Germany). Chlorostyrene and styrene oxide, and dicyclopentadiene were purchased from Aldrich (Steinheim, Germany) and Carl Roth (Karlsruhe, Germany). Chlorostyrene-catalase, formate dehydrogenase, and cofactors were purchased from Sigma-Aldrich (St. Louis, MO). D-thiogalactopyranoside). After 26 to 40 h of incubation (20°C), cells were harvested by centrifugation and disrupted in a precooled French press.

Expression and purification of recombinant StyA1 and StyA2B (R. opacus 1CP). Recombinant His6-StyA2B was expressed and purified as reported previously (40). The expression of His6-StyA2B was made in a bio-fermenter (71-L Bioreactor, ADI 1030 Biocellator; Applikon). For this purpose, 4 liters LB-medium (ampicillin and chloramphenicol) was inoculated with 50 ml of a preculture of E. coli BL21 (pSRoA1_P01), and cultivation was performed at 30°C (600 rpm, 0.3 standard liters of air per min) up to an optical density at 600 nm (OD600) of approximately 0.7. Temperature was then gradually decreased to 20°C and induction was started by adding 0.1 to 0.2 mM IPTG (isopropyl-β-D-thiogalactopyranoside). After 26 to 48 h of incubation (20°C), cells were harvested by centrifugation and disrupted in a precooled French press.

Expression, purification, and refolding of recombinant PheA2 (R. opacus 1CP). The oxidoreductase PheA2 is the small component of a flavin-dependent two-component phenol monooxygenase from R. opacus 1CP (our unpublished data), and it reduces FAD to FADH2 at the expense of NADH.

The attempt to improve the yield and turn expression from insoluble toward soluble protein failed (data not shown).

Results. Attempts (change expression vector, host, or conditions) to improve the expression and purification/refolding according to the instructions of the QuickFoldTM protein refolding kit (Athena Environmental Sciences, Inc., Baltimore, MD). Inclusion body pellets resulting from crude extract preparation were subjected to four washing steps using 50 mM Tris-HCl (pH 8.0), 4 M urea, 0.5 M NaCl, 1 mM EDTA, 0.1% (vol/vol) Triton X-100.

FIG. 1. Enantioselective oxygenations catalyzed by styrene monoxygenases (SMOs). Each * indicates a chiral atom in the formed products.

FIG. 2. Schemes for genetic organization and proposed mechanism of StyA/StyB from Pseudomonas sp. VLB120 and StyA1/StyA2B from R. opacus 1CP. For simplicity, only monomers are shown. (a) For Pseudomonas sp. VLB120, gene cluster encoding the upper styrene degradation pathway: StyC and StyR regulatory genes; StyA and StyB encoding a styrene monooxygenase, StyC encoding a styrene oxide isomerase, and styD encoding phenylacetaldelyde dehydrogenase (31). For R. opacus 1CP, organization of investigated styrene-catabolic genes: styA1 for putative monooxygenase component of a two-component SMO and styA2B for single-component SMO (40). (b) From left to right, cooperative mechanism of a typical two-component SMO of Pseudomonas (27), mechanism of the self-sufficient monooxygenase StyA2B of R. opicus 1CP (40), and hypothetical cooperation between StyA2B and StyA1. The FADH2 surplus of StyA2B is utilized by StyA1, yielding an increase of styrene-oxygenating activity. Dashed arrows indicate uncoupling-based FADH2 autooxidation leading to the formation of hydrogen peroxide.
Washed pellets were solubilized in 50 mM Tris HCl (pH 8.0), 8 M urea, 10 mM dithiothreitol (DTT) and incubated at 5°C until completely dissolved. Refolding of recombinant PheA2 was achieved by diluting ∼2 mg m l⁻³ solubilized enzyme dropwise 1:20 into 50 mM Tris HCl (pH 8.5), 240 mM NaCl, 10 mM KCl, 1 mM EDTA, 0.05% (wt/vol) PEG 4000, 1 mM glutathione reduced, 0.1 mM glutathione oxidized. The solution was carefully mixed by inversion and 20-min incubation at room temperature. Concentration of refolded protein was achieved by ammonium sulfate precipitation (80% final saturation). After 2.5 h of incubation on ice and subsequent centrifugation (20,000 g particle size, 100-Å m particle size, 100-Å mgCl₂ and 2 mM DTT and again centrifuged to remove remaining insoluble protein. Aliquots containing 50% (vol/vol) glycerol were stored at -20°C.

Expression, purification, and refolding of recombinant StyB (Pseudomonas sp. VLB120). Expression of recombinant StyB was carried out according to Otto and coworkers (27) with the following modifications: (i) expression was carried out in shaking flasks with 100 ml to 300 ml LB medium and appropriate amounts of antibiotics, (ii) E. coli BL21 pLYSs was used as a host harboring pTEZ302, and (iii) induction of the alk regulatory system of the expression vector was accomplished by adding 0.05% (vol/vol) octanol instead of diisopropylketone. Recombinant StyB was exclusively expressed as inclusion bodies.

Purification, washing, and refolding procedures of StyB inclusion bodies were performed as described previously (27). Buffer exchange for 25 mM Tris HCl, pH 7.5, 1 M NaCl, 0.5 mM MgCl₂, and 2 mM DTT and again centrifuged to remove remaining insoluble protein. Aliquots containing 50% (vol/vol) glycerol were stored at -20°C.

Protein quantification. Protein concentrations were determined with the Bradford method (8) using the protein assay reagent (Bio-Rad). Bovine serum albumin (Sigma) served as a reference protein. Alternatively, His_tagStyA2B concentrations were determined at 280 nm using a NanoDrop photometer (PegLab).

Oxidoreductase activity determination. Oxidoreductase activities of StyB from Pseudomonas sp. VLB120, PhaA2 from R. opacus 1CP, and StyA2B from R. opacus 1CP were determined by measurement of NADH consumption at 340 nm. A typical assay (1-ml total volume) contained 20 mM Tris HCl (pH 7.5), 0.05 to 0.15 μM FAD, and a suitable amount of enzyme. After equilibration at 30°C for 10 min, the reaction was started by addition of 0.175 μM NADH (40).

Oxygenase activity determination. The oxygenase activity of recombinant StyA1 toward styrene and structurally similar substrates was measured by reversed-phase high-pressure liquid chromatography (RP-HPLC)-based quantification of the reaction product using the assay system described for StyA2B (40). Reaction rates (measured at least in triplicate) were determined from the initial slope of product formation, and relative velocities were calculated by referring the obtained specific activities to that for styrene (100%). From kinetic data, a standard error of <20% was observed. To regenerate trapped oxygen from hydrogen peroxide formed via uncoupling of the reductase-oxygenase system, an oxygenase activity of recombinant StyA1 toward styrene and structurally similar substrates was measured by reversed-phase HPLC on a vertex column packed with Eurospher 120 (150 mM NaCl. A low- and high-molecular-weight reference protein kit (Amerham Pharmacia) was used for size calibration.

Proteinase activity. The proteinase activity of recombinant StyA1 was determined by size exclusion chromatography using a Superdex-200 HR16/60 column (GE Healthcare) running in 100 mM Tris HCl (pH 7.5), 150 mM NaCl. A low- and high-molecular-weight reference protein kit (Amerham Pharmacia) was used for size calibration.

Nucleotide sequence accession number. The 8,965-bp genomic fragment including the genes styA1 and styA2B from R. opacus 1CP has been deposited at GenBank under accession no. FJ403049 (40). The pheA2 sequence from strain 1CP has been deposited at GenBank under accession no. FN908432.

### RESULTS

Expression and hydrodynamic properties of recombinant StyA1. Recombinant Hist10-StyA1 was expressed in E. coli (pSRoA1_P01) mainly in the form of inclusion bodies. Active soluble protein (about 7.5 mg final yield) was obtained from a 4-liter bioreactor culture by keeping the temperature during...
might retrieve FADH₂ from StyA₂B, enabling its styrene epoxidation. The monooxygenase StyA₁, which is adjacent to the styB gene, was identified close to the styA₂B gene of R. opacus 1CP (40). Thus, the monooxygenase StyA₁ and the reductase StyB from Pseudomonas sp. VLB120 were independently determined. The oxygenase activity of StyA₁ was highest in the presence of StyB reductase from Pseudomonas sp. VLB120. This approach was chosen since the specificity between oxygenases and FAD reductases seems rather broad, especially for SMOs (27, 45).

Table 2: StyA₁ oxygenase activity in dependence of different FAD reductases

<table>
<thead>
<tr>
<th>FAD reductase</th>
<th>Molar amount (pmol)</th>
<th>FAD-reducing activity (mU)</th>
<th>StyA₁ oxygenase activity (mU)</th>
<th>Epoxidation activity (mU)</th>
<th>Relative efficiency (%)</th>
<th>Efficiency of oxygenation</th>
</tr>
</thead>
<tbody>
<tr>
<td>StyA₂B</td>
<td>62.5</td>
<td>13.2</td>
<td>125</td>
<td>0.58 (0.66)</td>
<td>46</td>
<td>0.044</td>
</tr>
<tr>
<td></td>
<td>125</td>
<td>26.4</td>
<td></td>
<td>1.28 (1.43)</td>
<td>100</td>
<td>0.049</td>
</tr>
<tr>
<td></td>
<td>250</td>
<td>52.8</td>
<td></td>
<td>1.22 (1.52)</td>
<td>96</td>
<td>0.023</td>
</tr>
<tr>
<td>StyB</td>
<td>62.5</td>
<td>67.2</td>
<td>125</td>
<td>2.06</td>
<td>161</td>
<td>0.031</td>
</tr>
<tr>
<td></td>
<td>125</td>
<td>135.5</td>
<td></td>
<td>2.03</td>
<td>159</td>
<td>0.015</td>
</tr>
<tr>
<td></td>
<td>250</td>
<td>271</td>
<td></td>
<td>1.11</td>
<td>87</td>
<td>0.004</td>
</tr>
<tr>
<td>PheA₂</td>
<td>250</td>
<td>87</td>
<td>250</td>
<td>0.18</td>
<td>7</td>
<td>0.002</td>
</tr>
</tbody>
</table>

* All experiments were performed in triplicate, and for oxygenations, a standard error of <20% was observed. Values in parentheses refer to experimentally obtained data comprising additional epoxidation activity of bifunctional StyA₂B (0.019 U mg⁻¹).
* Normalized values set to 100% for equimolar StyA₁/StyA₂B reaction (for 125 pmol StyA₁).
* Efficiency is defined as the ratio of oxygenase-generated epoxide per reductase-generated FADH₂.
* With PheA₂ as reductase, only higher amounts of StyA₁ yielded reliable epoxidation rates.

Expression and purification of other monooxygenase components. Recombinant His₉₅-StyA₂B showed a comparable FAD-reducing activity (3.3 U mg⁻¹) and higher oxygenase activity (0.02 U mg⁻¹) after purification as previously described (40). Recombinant PheA₂ from R. opacus 1CP, initially obtained as inclusion bodies, could be refolded to the active form with a specific FAD reductase activity of 20.7 U mg⁻¹. The protein yielded a single band of about 17 kDa during SDS-PAGE and behaved as a homodimer in gel filtration. The recombinant StyB from Pseudomonas sp. VLB120 was successfully expressed, purified, and refolded as described elsewhere (27). With a specific FAD-reducing activity of 60 U mg⁻¹, StyB appeared to be the most active reductase applied in this study.

Identification of the capability of StyA₁ for styrene epoxidation. Sequence analysis of StyA₁ from R. opacus 1CP revealed the highest similarities to several oxygenases of two-component styrene monooxygenases from pseudomonads (40). A gene coding for a single flavin reductase component could not be identified close to styA₁, an arrangement which is typical for these genes in pseudomonads (Fig. 2A) (26). Instead, an adjacent styB gene is part of styA₂B (Fig. 2A), which encodes a self-sufficient SMO (40). Thus, the monooxygenase StyA₁ might retrieve FADH₂ from StyA₂B, enabling its styrene epoxidation activity (Fig. 2B). Detection of StyA₁ activity in the presence of StyA₂B needs special consideration in view of the oxygenase activity of bifunctional StyA₂B. Therefore, we independently determined the oxygenase activity of StyA₁ in the presence of the StyB reductase from Pseudomonas sp. VLB120. This approach was chosen since the specificity growth phase at 30°C and by decreasing the temperature before induction to 20°C. SDS-PAGE of Ni-chelate-purified His₉₅-StyA₁ showed a single protein band with an expected molecular mass of ~44 kDa (not shown; theoretical molecular weight [MW], 46,537). No covalently bound flavin was detected via fluorescence analysis after SDS-PAGE separation. Elution behavior of the native protein during size exclusion chromatography pointed to a hydrodynamic size of 80 ± 5 kDa, which corresponds to a homodimer. A similar native structure was reported for StyA from R. opacus 1CP (40). Recombinant PheA₂ from R. opacus 1CP showed the highest activity for the StyA₁/StyB system (2.03 mU; this value corresponds to a specific activity of 0.35 U mg⁻¹), followed by StyA₁/StyA₂B (1.28 mU) and StyA₁/PheA₂ (0.09 mU) for 125 pmol of each protein (Table 2). Reliable epoxidation rates from the last combination were yielded only from 250 pmol of each component. The combinations of 125 pmol StyA₁ and 125 or 250 pmol PheA₂ did not yield a measurable amount of epoxide.

Activity of StyA₁ with different FAD reductases. StyA₁ activity showed a remarkable dependence on the type of FAD reductase. Comparison of equimolar ratios of both enzymes showed the highest activity for the StyA₁/StyB system (2.03 mU; this value corresponds to a specific activity of 0.35 U mg⁻¹), followed by StyA₁/StyA₂B (1.28 mU) and StyA₁/PheA₂ (0.09 mU) for 125 pmol of each protein (Table 2). Reliable epoxidation rates from the last combination were yielded only from 250 pmol of each component. The combinations of 125 pmol StyA₁ and 125 or 250 pmol PheA₂ did not yield a measurable amount of epoxide.

In view of the different specific activities of the reductases, the term epoxidation efficiency was introduced. This efficiency indicates the amount of FADH₂ cofactor necessary for the incorporation of one atom of oxygen in the process of styrene epoxidation. The very low activity of StyA₁ in the presence of equimolar amounts of PheA₂ points to a low degree of cooperation between these proteins. StyA₂B- and StyB-containing systems with lower or similar FAD-reducing capacities show a considerably higher epoxidation efficiency with StyA₁ (Table 2). The StyA₁/StyA₂B couple is clearly the most efficient.
epoxidation system, utilizing approximately 20 molecules of FADH₂ for the epoxidation of one molecule styrene. Both StyA2B surplus as well as StyA2B deficit result in a decrease of oxygenation activity compared to the equimolar mixture, thus indicating a highly balanced system. The observation that an increase of StyA2B does not bring about a further increase of StyA1 activity suggests that FADH₂ transfer is induced by a direct contact between the monooxygenase components rather than occurring as free diffusion. In this case, a higher StyA2B ratio should not lead to remarkable FADH₂ overproduction which traps oxygen and limits StyA1 activity, because StyA2B has rather low FAD-reducing activity compared to StyB (Table 2).

**Oxygenation specificity of StyA1.** The StyA1/StyA2B system was studied for its oxygenation specificity. For this purpose, both enzymes were mixed in equimolar amounts. Under these conditions, the epoxidation efficiency is maximal and StyA2B primarily acts as a FAD reductase (Table 2). Epoxidation was observed with styrene, 2-chlorostyrene, 3-chlorostyrene, 4-chlorostyrene, 4-methylstyrene, and dihydronaphthalene, while methylphenylsulfide is transformed into a sulfoxide (Table 3). Like StyA2B, StyA1 is able to convert a number of aromatic compounds. Comparison of relative StyA1 and StyA2B activities indicates a distinct selectivity which is especially obvious from the conversion of methylphenylsulfide, 3-chlorostyrene, and 2-chlorostyrene. The last compound is not a substrate for StyA1. As a consequence and depending on the type of substrate, StyA2B contributes different degrees of oxygenation activity to the StyA1/StyA2B system (Fig. 3). Compared to StyA2B alone, the StyA1/StyA2B system brings about a 10-fold increase of activity (with styrene as substrate), showing that the surplus of the FAD-reducing capacity of StyA2B can be efficiently used by StyA1.

The stereospecificity of StyA1 (enantioselective excess [e.e., >96%]) is comparable to that of StyA2B (e.e., >94%). Styrene is converted by both monooxygenases at the (S)-epoxide in a highly enantioselective manner, a property which is shared by all SMOs reported so far.

**Flavin binding of monooxygenase components.** Styrene monooxygenases contain a Rossmann fold for binding FAD via its AMP moiety (27, 40, 42). This and the fact that FADH₂ transfer is necessary for SMO activity raises the question of the protein-flavin interaction. TCA precipitation revealed that StyA1 and StyA2B both contain trace amounts of noncovalently bound FAD (1 to 4 moles percent [mol%]) (Fig. 4a). A similar result was obtained by spectrophotometrical analysis of StyA2B (40). This indicates that StyA1 and StyA2B have some affinity for oxidized FAD, since FAD was never added in any purification step.

To study the specificity of the protein-flavin interaction in more detail, the purified StyA1 and StyA2B apoproteins were titrated with FAD and analyzed for changes in tryptophan fluorescence. In the case of StyA1, the tryptophan fluorescence decreased considerably and clear binding of FAD was observed (Fig. 4b). From the titration curve and assuming 1:1 binding (0.59 μM StyA1), a Kᵣ value for FAD [Kᵣ(FAD)] of 0.23 ± 0.06 μM was estimated for the dissociation constant of the StyA1-FAD complex. In the case of StyA2B (0.66 μM; Fig. 4b), the tryptophan fluorescence decreased also upon FAD addition, but the curve shapes differed, and the final normalized tryptophan fluorescence was clearly lower than that from StyA1. This quenching behavior might result from the two flavin binding sites of StyA2B, one in the reductase and one in

### TABLE 3. Oxygenation specificity of StyA1 and other styrene monooxygenases

<table>
<thead>
<tr>
<th>Substrate/product</th>
<th>StyA1 of R. opacus 1CP and StyA2B of R. opacus 1CP&lt;sup&gt;a&lt;/sup&gt;</th>
<th>StyA2B of R. opacus 1CP and StyA2B of R. opacus 1CP&lt;sup&gt;c&lt;/sup&gt;</th>
<th>StyA1 + StyA2B of R. opacus 1CP&lt;sup&gt;d&lt;/sup&gt;</th>
<th>StyA of Pseudomonas sp. VLB120 and electrochemical FAD reduction&lt;sup&gt;e&lt;/sup&gt;</th>
<th>Unknown SMO, metagenome, and PheA2 of Geobacillus thermoglucosidius&lt;sup&gt;f&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Styrene/styrene oxide</td>
<td>0.214 (100) &gt;96 (S)</td>
<td>0.019 (100) &gt;94 (S)</td>
<td>0.233 (100) &gt;94 (S)</td>
<td>0.204 (100) 98 (S)</td>
<td>0.312 (153) 98 (S)</td>
</tr>
<tr>
<td>2-Chlorostyrene/2-chlorostyrene oxide</td>
<td>&lt;0.001 (&lt;0.1)</td>
<td>0.007 (37)</td>
<td>0.007 (3)</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>3-Chlorostyrene/3-chlorostyrene oxide</td>
<td>0.162 (76)</td>
<td>0.044 (222)</td>
<td>0.206 (88)</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>4-Chlorostyrene/4-chlorostyrene oxide</td>
<td>0.078 (36)</td>
<td>0.011 (58) &gt;82</td>
<td>0.109 (38)</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>4-Methylstyrene/4-methylstyrene oxide</td>
<td>0.102 (48)</td>
<td>0.006 (32)</td>
<td>0.108 (46)</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Dihydronaphthalene/dihydronaphthalene oxide</td>
<td>0.171 (80)</td>
<td>0.011 (58)</td>
<td>0.182 (78)</td>
<td>0.316 (184) 99 (S)</td>
<td>0.312 (153) 98 (S)</td>
</tr>
<tr>
<td>Methylphenylsulfide/methylphenylsulfoxide</td>
<td>0.325 (152)</td>
<td>0.012 (63)</td>
<td>0.337 (145)</td>
<td>0.226 (111) 26 (11)</td>
<td>0.11 (14) 75 (R)</td>
</tr>
</tbody>
</table>

<sup>a</sup> Values for StyA1 were obtained from total activities of StyA1/StyA2B by subtracting oxygenation rates of StyA2B.
<sup>b</sup> ND, not determined.
<sup>c</sup> This study and reference 40.
<sup>d</sup> Reference 17. A maximum activity of 2.1 U mg⁻¹ for the recombinant StyA/StyB system was reported later (27).
<sup>e</sup> Reference 45.
the oxygenase domain. Assuming one binding site, we estimated a $K_d(FAD)$ of $5.05 \pm 0.52 \mu M$ for the StyA2B-FAD complex. Assuming two independent binding sites, we estimated the following dissociation constants: $K_d(FAD)$ of $0.05 \pm 0.17 \mu M$ and $K_d(FAD)$ of $7.19 \pm 0.61 \mu M$. These values have to be taken with care, since cooperativity cannot be excluded. Furthermore, the very small contribution of the first binding site ($<5\%$) to the total decrease of the tryptophan fluorescence and the high standard error for $K_d(FAD)$ indicates another behavior. Thus, the dissociation constants given herein for StyA2B indicate only the $\mu M$ range of both values.

**DISCUSSION**

The StyA1/StyA2B system reported herein represents a new type of FAD-dependent styrene monooxygenase (SMO). Recently, we described StyA2B from *R. opacus* 1CP as the first self-sufficient SMO (40). StyA2B comprises both styrene-oxidizing and FAD-reducing activity in one polypeptide and has a substrate pattern similar to those of conventional two-component SMOs (Table 3). However, the specific activity of StyA2B is rather low ($0.02 \mu g^{-1}$), and the FADH$_2$ formed by the reductase domain, is inefficiently used by the fused oxygenase domain. These observations and the fact that an additional *styA1* gene is located directly upstream from *styA2B* raised the hypothesis about a functional interaction between StyA1 and StyA2B (Fig. 2).

The His-tagged StyA1 protein was successfully expressed in *E. coli* and appeared to be a homodimer of about 80 kDa. Activity measurements with StyB from *Pseudomonas* sp. VLB120 showed that StyA1 is able to oxidize styrene under consumption of FADH$_2$. When StyB was replaced by PheA2 from *R. opacus* 1CP, a drastic decrease in StyA1 activity occurred (Table 2). This is rather surprising because the FADH$_2$ production rate of PheA2 (20.7 $\mu g^{-1}$) is similar to that of StyB (60 $\mu g^{-1}$). For the prototype StyA/StyB monooxygenase from *Pseudomonas* sp. VLB120, it was shown that the StyB reductase component can be replaced by another FAD reductase (27) or by an electrochemical reductant (Fig. 2b) (16, 17). From this it was argued that the FADH$_2$ needed for styrene epoxidation is provided via free diffusion and that no specific interaction between StyA and StyB is needed. However, an equimolar amount of PheA2 achieved only 4 to 7% of StyA1 activity compared to that of assays with StyB or StyA2B. This suggests that free FADH$_2$ is not feasible for StyA1 and that efficient epoxidation requires some type of interprotein communication. An alternative, less likely explanation is that the StyA1 activity is inhibited by PheA2.

With the StyA1/StyA2B system, the highest StyA1 activity was observed at an equimolar ratio of both components (Table 2). Bisection of the StyA2B amount and therewith reductase power yielded half of the StyA1 activity. Moreover, a surplus of the StyA2B amount and a similarly enhanced reductase power does not increase StyA1 activity. These observations support the suggestion that StyA1 is not active with free FADH$_2$ and recognizes StyA2B as its natural partner. Similar transfer mechanisms based on transient interactions or complex formation have been proposed for other two-component flavin monooxygenases (1, 9, 10, 18). Based on the present findings, a putative StyA1/StyA2B reaction mechanism can be postulated (Fig. 2b). In this mechanism, both dimeric protein components form a transient complex in which FADH$_2$ is channeled from StyA2B toward StyA1, resulting in epoxide formation. As side reactions, some epoxide is formed in the oxygenase-active site of StyA2B and H$_2$O$_2$ is produced due to auto-oxidation of FADH$_2$. This reaction mechanism differs from that of conventional SMOs, especially in FADH$_2$ transfer, since it is induced by protein cross talk. Nevertheless, it becomes obvious and must be kept in mind that the system of StyA1/StyA2B described herein is still less efficient in flavin transfer since 20 FADH$_2$ molecules are necessary to form one molecule of epoxide (Table 2). Unfortunately, no data for the StyA1/StyA2B system in the natural host *R. opacus* 1CP are available.

Binding studies showed that StyA1 and StyA2B interact with oxidized FAD (Fig. 4). In the case of StyA2B, tryptophan fluorescence quenching pointed to two FAD binding sites with
different cofactor affinities (strong, $K_{d1} = 0.05 \pm 0.17 \mu M$; and weak, $K_{d2} = 7.19 \pm 0.61 \mu M$) for StyA ($K_d = 21.2 \pm 2 \mu M$) and StyB ($K_d = 2.3 \pm 0.3 \mu M$) from *Pseudomonas* sp. VLBI20, the reductase component had a higher affinity for FAD than the oxygenase component (27). Thus, it might be argued that the relatively strong interaction between StyA2B and FAD is caused by the reductase domain and not by the Rossmann fold of the oxygenase domain. This is further supported by the fact that the related PheA2 reductase from *Geobacillus thermoglucosidasius* binds FAD with nanomolar affinity (20, 44).

StyA1 from *R. opacus* 1CP binds oxidized FAD ($K_d = 0.23 \pm 0.06 \mu M$) two orders of magnitude better than StyA from *Pseudomonas* sp. VLBI20 ($K_d = 21.2 \pm 2 \mu M$; 27). Nevertheless, the affinity of StyA1 to FAD is consistent with the fact that SMOs have a strong preference for binding FADH$_2$ (19, 42). For StyA from *Pseudomonas putida* S12, it was reported that the reduced flavin cofactor binds about 8,000 times more tightly than the oxidized form (42). Based on this and structural considerations, a kinetic mechanism was proposed in which the reaction of oxygen with StyA-bound FADH$_2$ precedes the binding of styrene. In the case of the StyA1/StyA2B system, we cannot exclude another sequence of events, but the herein-described multifunctional monooxygenase StyA1/StyA2B needs further investigation and is an attractive target for optimization for biotechnological purposes.

In conclusion, StyA1/StyA2B from *Rhodococcus opacus* 1CP is a novel, unusual member of the family of two-component flavin-dependent monooxygenases (10, 43). The raised hypothesis of a functional interaction of both proteins was reinforced. StyA1 was identified as the major monooxygenase, and StyA2B turned out to function mainly as a FAD reductase with little oxygenating side activity. The StyA1/StyA2B system has an overall epoxidation activity of 0.24 U mg$^{-1}$, which competes favorably with the activity of previously described SMOs (27, 45). Why has nature designed such a complex monooxygenase system? It cannot be excluded that the occurrence of the StyA2B fusion is a survival or intermediate from evolutionary events. No activity data of a comparable system are available yet. Only three sets of genes homologous to *stya*-*stya2B* are known. All are adjacently located in genomes of Gram-positive actinobacteria (*Arthrobacter*, Nocardia, and Streptomyces) and share 50 to 70% amino acid sequence similarities (40). Indeed, the herein-described multifunctional monooxygenase StyA1/StyA2B needs further investigation and is an attractive target for optimization for biotechnological purposes.

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**References**


23. Mooney, A., P. G. Ward, and K. E. O’Connor. 2006. Microbial degradation of aromatic hydroxylases (11). StyA1 shows a significant affinity for FAD and may bind FAD if no FADH$_2$ is transferred (Fig. 4b). Binding of the oxidized flavin cofactor might be beneficial for the protein stability (7, 15), but it may also inhibit the monooxygenase activity by competing with FADH$_2$ for the flavin binding site. The (transient) interaction between StyA1 and StyA2B might change the affinity of StyA1 for FAD(H)$_2$. However, as noted above, it is more likely that it supports the exchange and limits the uncoupling of epoxidation (Table 2).

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