Functional and Structural Characterization of a Cation-dependent O-Methyltransferase from the Cyanobacterium Synechocystis sp. Strain PCC 6803**§

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The coding sequence of the cyanobacterium Synechocystis sp. strain PCC 6803 slr0095 gene was cloned and functionally expressed in Escherichia coli. The corresponding enzyme was classified as a cation- and S-adenosyl-l-methionine-dependent O-methyltransferase (SynOMT), consistent with considerable amino acid sequence identities to eukaryotic O-methyltransferases (OMTs). The substrate specificity of SynOMT was similar with those of plant and mammalian CCoAOMT-like proteins accepting a variety of hydroxycinnamic acids and flavonoids as substrates. In contrast to the known mammalian and plant enzymes, which exclusively methylate the meta-hydroxyl position of aromatic di- and trihydroxy systems, SynOMT also methylates the para-position of hydroxycinnamic acids like 5-hydroxyferulic and 3,4,5-trihydroxycinnamic acid, resulting in the formation of novel compounds. The x-ray structure of SynOMT indicates that the active site allows for two alternative orientations of the hydroxylated substrates in comparison to the active sites of animal and plant enzymes, consistent with the observed preferred para-methylation and position promiscuity. Lys3 close to the N terminus of the recombinant protein appears to play a key role in the activity of the enzyme. The possible implications of these results with respect to modifications of precursors of polymers like lignin are discussed.

Methylation by S-adenosyl-l-methionine (AdoMet)-dependent O-methyltransferases (OMTs)2 (EC 2.1.1) is a common modification in secondary product biosynthesis (1). Site-specific O-methylation modulates the physiological properties and the chemical reactivity of phenolic compounds and renders them more hydrophobic. Cation-dependent OMTs constitute a small group of low molecular mass (23 to 27 kDa) enzymes (2). In mammals, these enzymes play important roles in the modification of catechol neurotransmitters in the brain or may inactivate potentially bioactive metabolites like quercetin in the liver and kidney (3, 4). They are therefore referred to as catechol OMTs (COMT) and have been investigated as potential targets to cure degenerative brain diseases (5). In plants, caffeoyl-coenzyme A O-methyltransferases (CCoAOMTs), named after their preferred in vitro substrate, in conjunction with a second group of cation-independent caffeic acid OMTs, are crucial for determining the structural integrity of lignin in plant vascular tissues (6, 7). Specific subtypes of CCoAOMT-like proteins also methylate, besides caffeoyl-CoA, other phenylpropanoids, preferentially flavonoids, with vicinal dihydroxy groups (2). The three-dimensional structures of eukaryotic animal and plant OMTs known so far are quite similar despite their otherwise low sequence identities, irrespective of the involvement of bivalent cations and substrates. Structural data obtained so far reveal a conserved AdoMet-binding site in all OMTs from the animal and plant kingdom (8–10). The methyl transfer mechanism proceeds via an S′-2-like transition state and a cation-facilitated deprotonation of one of the two hydroxyl groups. Atomic structures of the cation-dependent catechol OMT from rat (11) and the two groups of plant CCoAOMTs (10, 12) have been solved. Although the structural details and the substrate preferences of each enzyme subtype vary, their corresponding position specificity toward the meta-position in aromatic structures is preferred. In plants, methylation is directed only toward the meta-position, whereas in mammals this preference is less stringent in case of the membrane-bound isomor (4). This scenario appears to be different in prokaryotes. In all prokaryotes analyzed so far and deposited in the databases (with the exception of archaeobacteria), at least one member of CCoAOMT-like proteins is present. In a recent report, the product of the SafC gene, a catechol OMT-like protein involved in methylation of the isoquinoline derivative safamycin from the prokaryote Myxococcus xanthus, was described as methylating the 4′-hydroxyl position of L-dihydroxyphenylalanine and of other catechols, such as caffeic acid with a ratio of 3:1 in favor...
of the para-position (13). The structure of another prokaryotic protein from the human pathogen *Leptospira interrogans* was also described, although neither the cation dependence nor any functional data are known (14).

In this article we report cloning, functional expression, enzymatic characterization, and x-ray structure elucidation of a CCoAOMT-like enzyme from the cyanobacterium *Synechocystis* sp. strain PCC 6803. In contrast to all known eukaryotic enzymes, this OMT displays a promiscuous position specificity favoring the para-hydroxyl group of polyhydroxylated aromatic phenolics for the attachment of the methyl moiety. Data are discussed with respect to structure differences of the active site, evolution, and application of this new enzyme activity in the design of aromatic mono- and polymers.

**EXPERIMENTAL PROCEDURES**

**Growth of Synechocystis sp. Strain PCC 6803 and DNA Isolation**—The glucose-tolerant strain of *Synechocystis* sp. PCC 6803 was obtained from N. Murata (National Institute for Basic Biology, Okazaki, Japan) and grown photoautotrophically in batch cultures at 29 °C under continuous illumination of 130 μmol of photons s⁻¹ m⁻² (warm light, Osram L58 W32/3) with bubbling of air enriched with CO₂ (5%) in BG11 medium at pH 8.0. High molecular DNA was obtained by lysozyme action on the cells, purification with cetyltrimethylammonium bromide, followed by phenol/chloroform treatments as described (15).

**Isolation of the slr0095 DNA Sequence and Functional Expression**—The slr0095 coding sequence was amplified by PCR using *Pfu* polymerase (Promega) with the following primers: N-terminal primer 5'-CGGGATCCCATGGGTAAGGGC-3'; C-terminal reverse primer 5'-CTCAAGCTTGTCATATTTTGGATTTGCCA-3'. Subsequently the fragment was cut with BamHI and HindIII, ligated into the multicloning site of the pQE30 expression vector (Qiagen), and transformed into chemically competent *Escherichia coli* M15rep cells (Qiagen). Inducing the bacteria with 1 mM isopropyl-1-thio-β-D-galactopyranoside at exponential growth and harvesting 3 h after induction lead to accumulation of a His-tagged fusion protein with a yield of about 1 mg of soluble Slr0095 protein/liter of *E. coli* culture. The protein was purified by metal affinity chromatography using the Talon matrix (Clontech), checked for purity by SDS-PAGE, and rebuffered into 20 mM Tris/HCl, pH 7.5, by ultrafiltration (Millipore Waters). For determination of the molecular mass, size exclusion chromatography was performed on Superdex 200 (GE Healthcare) in 20 mM Tris/HCl, 150 mM NaCl, pH 7.5. Crystallization trials and all assays were performed with fresh protein preparations, because the active enzyme was unstable and could be stored for prolonged periods at a concentration of 1 mg/ml only with the inclusion of 10% PEG 400. Even then, after 2 months at -20 °C a 20% loss of activity was observed.

**Structural Characterization of the Cation-dependent SynOMT**—Purified recombinant SynOMT was set up for crystallization with a standard set of buffers. Crystals were obtained from 0.2 M MgCl₂, 0.1 M Tris/HCl, pH 8.5, 30% polyethylene glycol 4000 (w/v). Diffraction data were collected on a MSC Rigaku Raxis IV + diffractometer at a wavelength of 1.54 Å. The crystals diffracted to 2.0 Å and belong to the space group P3₁2₁ with cell constants of \( a = b = 57.62 \) Å and \( c = 119.83 \) Å. Integration and scaling of the diffraction data were performed using the HKL2000 software package (16). Analysis of the intensity distribution indicated merohedral twinning, with a twinning fraction of 0.284 (twinning operator \( h, -k, l \)) determined using CNS (17). The structure of SynOMT was solved by molecular replacement with the monomer of *M. crystallinum* PFOMT (12) (PDB ID code 3C3Y) using the program Phaser (18), part of the CCP4 suite (19), revealing one molecule in the asymmetric unit. Manual rebuilding of the model was performed with the use of the programs O (20) and Coot (21). The structure was refined using CNS to an R = 0.152 and twinned \( R_{	ext{free}} = 0.215 \). Data statistics are given in Table 4. The structure has been assigned the PDB ID code 3CBG. The bootstrapped phylogenetic cladogram was constructed using the TREECON software tools (22).

**Activity Tests and Kinetic Data**—Enzyme assays were performed 100 mM potassium phosphate buffer, pH 7.5, and 10% glycerol with 10 μM substrate (dissolved in 30% dimethyl sulfoxide), 0.5–2 μg of total protein, and 400 μM AdoMet in a total volume of 50 μl. Assays were incubated at 30 °C for 60 to 3600 s (dependent on the protein and substrate tested) and stopped by the addition of 20 μl of 7% trichloroacetic acid in 50% acetonitrile/water. The reaction products were analyzed by HPLC as described (2). Caffeoyl glucose was prepared as described previously (23) from caffeic acid and UDP-glucose with the purified recombinant sinapic acid glucosyltransferase. Caffeoyl-CoA was prepared based on published methods (24, 25).

Caffeic acid was obtained from Serva, quercetin from Extrasythése, and quercetin from Roth, respectively. Tricetin was a generous gift from Ragai Ibrahim (Montreal, Canada). All substrates and reaction products were analyzed by RP-HPLC on a Nucleosil 5-μm C₁₈ column (50 mm length × 4 mm inner diameter; Macherey & Nagel), as described previously (2). Compounds were analyzed with linear gradients from 10% B (acetonitrile) in A (1.5% aqueous phosphoric acid) to 70% B in A (for phenolics), from 5% B to 50% B in A (for free acids and CoA esters), from 5% B to 30% B (for glucose esters), and from 20% B to 10% A to 80% B in A (for flavonoids) in 4 min at a flow rate of 1 ml/min. UV detection of flavonoids, catechol, coumarins, and hydroxycinnamic acid esters was performed between 200 and 400 nm. Identification and quantification was achieved with reference compounds from our institute collection. For \( K_m \) determination of methyl group acceptors, acceptor concentrations were chosen between 2 and 40 μM, whereas AdoMet was kept constant at 1.5 mM. The \( K_m \) and \( V_{\text{max}} \) values were calculated from Lineweaver-Burk plots (26). All enzyme assays were recorded from at least two different experiments each in triplicate.

**Identification of Methylation Products of 3,4,5-Trihydroxycinnamic Acid**—SynOMT reactions were run in 50-ml Falcon tubes at 37 °C with up to 5 mg of purified enzyme. Reactions were run for 60 min, centrifuged at 10,000 × g for 10 min, and the supernatants were applied directly to C₁₈-reversed phase solid phase extraction columns (Millipore Waters), washed with 5 column volumes deionized water, and the compounds were eluted with MeOH. The reaction products were further purified by RP-HPLC on a Nucleosil 5-μm C₁₈ column.
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at 3 ml/min (250 × 8 mm, inner diameter; Macherey & Nagel) with a linear gradient from 10% B (MeOH) in A (0.2% aqueous acetic acid) to 60% B in A over 30 min. Fractions from several runs were combined, concentrated, and subjected to NMR spectroscopy and mass spectrometry.

1H and homo- and heteronuclear two-dimensional NMR spectra from the major products dissolved in CD3OD were recorded on a Inova 500 NMR spectrometer (Varian) operating at 499.81 MHz using a 3-mm microsample inverse detection probe. Chemical shifts are referenced to internal TMS (δ = 0 ppm, 1H) and CD3OD (δ = 49.0 ppm, 13C), respectively (Table 1). Negative ion electrospray (ESI-MS) high-resolution mass spectra were obtained from a BRUKER Apex III Fourier Transform ion cyclotron resonance mass spectrometer equipped with an external APOLLO electrospray ion source: trihydroxyform ion cyclotron resonance mass spectrometer equipped with an external APOLLO electrospray ion source: trihydroxyform ion cyclotron resonance mass spectrometer equipped.

Gas chromatography (GC)/time-of-flight (TOF)-mass spectrometry (GC) measurements of several hydroxycinnamic acid derivatives and corresponding enzymatically methylated products were performed with an 6890 series gas chromatograph (Agilent) equipped with an autosampler 7683 series and a MicroMass GCT with a TOF detector (Waters) as recently described (27) (see Table 2).

Screening and Docking of Natural Products—A pharmacophore model of the active site was generated using MOE (28) based on the x-ray structure of SynOMT including distinct binding modes of ferulic and isoferrulic acids. The model includes the three hydrophilic binding sites related to the phenolic hydroxyl or methoxy groups, the center of the aramate, and a hydrophilic area defined by the carboxylic acid group of the ligands. All amino acid residues of the active site were used to define an excluded volume, so that no spatial overlap of any ligand with this region was allowed. This model was used to screen the MOE (28) and KEGG (29) databases for compounds containing three-dimensional structures with low energy conformations. Highly significant hits were docked to the active site using PLANTS (30).

RESULTS

Functional Characterization of Recombinant slr0095 Protein—Slr0095 represents the only gene identified in the Synechocystis sp. strain PCC 6803 genome coding for a protein with a significant identity to cation-dependent OMTs from animals and plants. The genes in the immediate neighborhood of slr0095, a putative heat shock protein and a sulfate transporter, gave no definite hint to the potential function of the gene in vivo (Fig. 1). Similar genes are found in a large number of prokaryotic genomes. Based on sequence identity searches in various databases, more than 200 genes from different prokaryotic organisms were found when complete or partial sequence motifs of plant CCoAOMT were used as in silico probes. Sequence identity to plant CCoAOMTs was much higher (up to 42% at the protein level) than to mammalian COMTs or bacterial CCoAOMT-like proteins (data not shown). A small subset and clustering of eukaryotic and prokaryotic sequences is displayed in Fig. 2. Although the prokaryotic sequences of CCoAOMT-like proteins, including Synechocystis slr0095, are distinct from plant sequences and appear to cluster together, the low bootstrap values do not allow the phylogenetic relationships among the prokaryotic sequences and non-plant eukaryotic sequences to be clearly defined.

The His-tagged and metal affinity purified SynOMT displayed a molecular mass of about 25 kDa on SDS-PAGE, corresponding to the predicted mass of the monomeric unit based on the amino acid sequence (Fig. 3). Using size exclusion chromatography under non-denaturing conditions, a dimeric structure with a calculated molecular mass of about 50 kDa appears likely (data not shown). In this respect the cyanobacterial SynOMT resembles the dimeric plant proteins rather than the animal COMTs, generally described as monomeric (10–12).

Characterization of Enzymatic Activities and Kinetic Measurements—Plant and mammalian cation-dependent OMTs in principle methylate the same type of substrates, i.e. aromatic compounds with vicinal di- or trihydroxy systems. The observed sequence identity and the presence of the characteristic AdoMet-binding domains suggested that the cyanobacterial OMTs would use the same or a similar set of compounds in vitro. SynOMT displayed activity against a variety of substrates (Table 3). Cation dependence was verified by inclu-
Notice that all bootstrap values below 500 do not allow phylogenetic relationships to be clearly defined. The neigh-
bor joining tree was based on 1000 bootstrap trials. The cluster of six plant proteins marked as CCoAOMTs is specific for caffeine-coenzyme A methylation in lignin monomer biosynthesis. Gene products of the cluster of six plant proteins marked as CCoAOMTs is specific for caffeoyl-coenzyme A methylation in lignin monomer biosynthesis. Gene products of Arabidopsis CCoAOMTs are listed based on the corresponding gene identifiers. Crystallized proteins are underlined. The mammalian catechol OMT from rat was used as an outgroup. NCBI data base accession numbers used are: BAA10567 (Synechocystis PCC 6803 slr 0095); NP_710596 (L. interrogans); Q00719 (S. mycarofaciens); BAB76778 (M. sativa CCoAOMT); AAD02050 (M. sativa CCoAOMT); AY057554, AAN61972 (Arabidopsis At1g34050), P22734 (rat catechol OMT).

FIGURE 2. Bootstraped cladogram of selected CCoAOMTs and CCoAOMT-like proteins from pro- and eu- karyotes as produced by TREECON (22). The neighbor joining tree was based on 1000 bootstrap trials. Notice that all bootstrap values below 500 do not allow phylogenetic relationships to be clearly defined. The cluster of six plant proteins marked as CCoAOMTs is specific for caffeoyl-coenzyme A methylation in lignin monomer biosynthesis. Gene products of Arabidopsis CCoAOMTs are listed based on the corresponding gene identifiers. Crystallized proteins are underlined. The mammalian catechol OMT from rat was used as an outgroup. NCBI data base accession numbers used are: BAA10567 (Synechocystis PCC 6803 slr 0095); NP_710596 (L. interrogans); Q00719 (S. mycarofaciens); BAB76778 (M. sativa CCoAOMT); AAD02050 (M. sativa CCoAOMT); AY057554, AAN61972 (Arabidopsis At1g34050), P22734 (rat catechol OMT).

FIGURE 3. SDS-PAGE demonstrating the purification of SynOMT. Lane 1, crude E. coli extract 4 h after isopropyl 1-thio-
galactopyranoside induction; lane 2, affinity column run-through; lane 3, wash fraction; lane 4, purified SynOMT fraction; lane 5, molecular mass ladder. The position of the SynOMT is marked with an arrow.

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sion or absence of EDTA in the buffers. Using 3,4,5-trihy- droxycinnamic acid and 5-hydroxyferulic acid as substrates, a novel product profile was observed by HPLC (Fig. 4A). 5-Hydroxy-
ferulic acid was methylated to sinapic acid and an additional unknown compound, whereas 3,4,5-trihydroxycinnamic acid and sinapic acid gave two peaks that could not be correlated to known standards by HPLC coupled to a diode-array UV-detect-
tion system (Fig. 4A). Absorbance maxima of the newly found products in the near UV were at 308 nm, inconsistent with any hydroxyci-
namic acid derivatives described so far. The products of 3,4,5-trihy-
droxycinnamic acid methylation catalyzed by a typical plant CCoAOMT-like protein, PFOMT from Mesembryanthemum crys-
tallinum (2), 5-hydroxyferulic and sinapic acid, display characteristic absorbance maxima between 320 and 325 nm (Fig. 4B). The lack of product formation by SynOMT with p-coumaric, ferulic, or sinapic acids ruled out that the transfer of the methyl group could be directed toward the carboxyl group of the phenylpropanoids, initially considered as a plausible alternative. For identification of the novel major product, NMR spectroscopy and ESI-MS data as well as GC/TOFMS data were combined with the UV data and expected specificities of cation-dependent OMTs (Tables 1 and 2). Combined use of one- and two-dimensional NMR experiments resulted in an unambiguous assignment of all 1H and 13C NMR signals of 3,5-dihydroxy-4-methoxycinnamic acid (with the exception of the 13C signal of –COOH, for sensitivity reasons). Only a 4-O-
methylation is in accordance with the isochronous NMR sig-
nals of positions 2/6 and 3/5. Furthermore, the para-position of the O-methyl group is proved by the high-field shift of C-4 (δ 13C 139.0 ppm), caused by the two-electron releasing hydroxyl substituents in ortho-position to C-4. In addition, no NOE enhancement is observed between the signal of the methoxyl group and the aromatic protons. These data are only consistent assuming an unusual preference of this enzyme for the para-
position, leading to the formation of 3,5-dihydroxy-4-methoxy-
cinnamic acid (PI in Fig. 5). The simultaneous appearance of two dimethylated compounds in addition to 3,5-dihydroxy-4-
methoxycinnamic acid was identified as sinapic acid, display characteristic spectral properties and retention times in HPLC as well as GC-MS. The second compound with identical spectral properties to the para-methylated 3,4,5-trihydroxycinnamic acid showed different LC and GC retention times compared with sinapic acid (Table 2, Figs. 4A and 5A). One compound was identified as 3,5-dimethoxy-4-hydroxycinnamic acid (sinapic acid) according to spectral properties and retention times in HPLC as well as GC-MS. The second compound with identical spectral properties to the para-methylated 3,4,5-trihydroxycinnamic acid showed different LC and GC retention times compared with sinapic acid (Table 2, Figs. 4B and 5B) and, corroborated by GC-MS data were thus identified as 5-hy-
droxy-3,4-dimethoxycinnamic acid. Consequently, incubation of SynOMT with 5-hydroxyferulic acid, consistent with previous results, resulted in the formation of 5-hydroxy-3,4-dime-
thoxycinnamic acid as well as sinapic acid as expected (Fig. 5A). This corroborates the observed specificity of CCoAOMT-like
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FIGURE 4. HPLC pattern of SynOMT-enzyme assays. Assays measured at 318 nm illustrate the methylation profiles (left) obtained with the substrate 3,4,5-trihydroxycinnamic acid either by the Sln0095 protein from Synechocystis sp. strain PCC 6803 (A) or the PFOMT from M. crystallinum (B) (2). S, substrate; P1, 3,5-dihydroxy-4-methoxycinnamic acid; P2, 3,5-dimethoxy-4-hydroxycinnamic acid (sinic acid); P3, 5-hydroxy-3,4-dimethoxycinnamic acid; P4, 3-methoxy-4,5-dihydroxycinnamic acid (5-hydroxyferulic acid).

TABLE 1

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* Chemical shifts of HSQC and HMBC correlation peaks.
* ND, not determined.

proteins for vicinal dihydroxy groups, but also indicates an apparent lack of position specificity. If a 3,4,5-trihydroxycinnamic acid molecule is methylated at the para-position, it does not undergo any further methylations, due to the absence of additional vicinal dihydroxy groups resulting in the major monomethylated product (Fig. 5A). In a second scenario the 3,4,5-trihydroxycinnamic acid is initially methylated at the meta-position, which is further methylated either at the para- or the second meta-position, resulting in a 50% ratio of 3,4- and 3,5-di-O-methylated products, respectively (Fig. 5A). When trihydroxylated flavonoids like the 3,3',4',5',5',7-hexa-hydroxylavone (myricetin) and the 3',4',5',5',7-pentahydroxyflavone (tricetin) were used as substrates, the enzyme displayed a higher position specificity, resulting only in the single 4'-O-methylated product (data not shown). This observation again is in contrast to the results for the corresponding plant enzymes, where two products were always observed with myricetin, tricetin, and 3,4,5-trihydroxycinnamic acid, consistent with methylation in the two meta-positions only (2).

Kinetic data of the array of substrates tested indicated that the trihydroxylated flavone tricetin is the best substrate (Table 3). Caffeoylglucose and especially 3,4-dihydroxybenzoic acid show a reduced affinity toward the enzyme, although their catalytic efficiencies in vitro are fairly similar to the best transformed substrates. In the case of 3,4-dihydroxybenzoic acid, the formation of 3-methoxy-4-hydroxybenzoic acid (vanillic acid) takes place at a 10-fold higher rate than the formation of 4-methoxy-3-hydroxybenzoic acid (isovanillic acid) as calculated from the peak areas in the GC-MS. It should be noted, however, that the substrates used in this study represent either synthetic compounds (3,4,5-trihydroxycinnamic acid) or are of plant origin and that the in vivo substrates of the SynOMT remain to be identified.

Structural Characterization of the Cation-dependent SynOMT—Crystals of SynOMT diffracting up to 2.0 Å (Table 4) were obtained in the presence of Mg\(^{2+}\), AdoMet, and caffeic acid. Inspection of the electron density clearly revealed the presence of AdoHcy and two methylation products, ferulic and isofurulic acid, in the active site of the enzyme (Fig. 6). The overall structure of SynOMT is that of a compact dimer whose fold closely resembles that of the plant CCoAOMTs. As described for Medicago sativa CCoAOMT (10) and M. crystallinum PFOMT (12), cyanobacterial SynOMT possesses the highly conserved \( \alpha/\beta \) Rossmann fold contributing significantly to the dimer interface. SynOMT differs from its plant counterparts in the disposition of the N terminus and in the conformation of the “insertion loop.” These regions, that are also among the most divergent regions of the divergent metal OMTs (supplemental Fig. S1), have been implicated in substrate specificity, in particular for caffeoyl-CoA (12).

The residues Asp\(^{143}\), Asp\(^{169}\), and Asp\(^{170}\), responsible for metal coordination, are conserved among the Mg\(^{2+}\)-dependent OMTs, as is the presence of a mediating water molecule that makes hydrogen bonds to the carbonyl oxygen of Met\(^{142}\) and the amide nitrogen of Ile\(^{44}\). The latter residue abuts the carboxylate group of AdoHcy. Interestingly, the equivalent of Ile\(^{44}\) is a threonine residue in the plant enzymes (supplemental Fig. S1), whose O\(^{-}\)is involved in a water-mediated hydrogen bond to the Mg\(^{2+}\) ion as well as a direct hydrogen bond to the AdoMet/AdoHcy carboxylate moiety, resulting in a slight displacement of the cofactor C\(^{\alpha}\) atom. As this residue is aliphatic in the bacterial enzymes, the small displacement as well as changes in the electronic characteristics of the metal ion could cause subtle differences in substrate specificity. Otherwise, cofactor binding is conserved among all the metal-dependent OMTs studied to date, involving hydrogen bonds to Gly\(^{68}\), Asp\(^{92}\), Ala\(^{121}\), and Asp\(^{145}\). The two products, ferulic and isofurulic acid, methylated in the meta- and para-positions, respectively, are clearly delineated in the electron density (Fig. 6B). The proponic acid...
TABLE 2
GC/TOFMS data of derivatized hydroxycinnamic acid standards and products of 3,4,5-trihydroxycinnamic acid methylation catalyzed by SynOMT

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<th>Identifier</th>
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TABLE 3
Kinetic parameters of the recombinant CCoAOMT-like SynOMT from Synechocystis sp. strain PCC6803

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<thead>
<tr>
<th>Substrates</th>
<th>K&lt;sub&gt;m&lt;/sub&gt; (µM)</th>
<th>k&lt;sub&gt;cat&lt;/sub&gt; (s&lt;sup&gt;-1&lt;/sup&gt;)</th>
<th>k&lt;sub&gt;cat&lt;/sub&gt;/K&lt;sub&gt;m&lt;/sub&gt; (s&lt;sup&gt;-1&lt;/sup&gt; µM&lt;sup&gt;-1&lt;/sup&gt;)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-Hydroxyferulic acid</td>
<td>74.0</td>
<td>0.02500</td>
<td>340</td>
</tr>
<tr>
<td>Caffeic acid</td>
<td>69.3</td>
<td>0.00551</td>
<td>80</td>
</tr>
<tr>
<td>Caffeoyl-CoA</td>
<td>32.9</td>
<td>0.02450</td>
<td>740</td>
</tr>
<tr>
<td>Caffeoylglucose</td>
<td>106</td>
<td>0.00951</td>
<td>90</td>
</tr>
<tr>
<td>3,4,5-Trihydroxycinnamic acid</td>
<td>20.7</td>
<td>0.01040</td>
<td>500</td>
</tr>
<tr>
<td>Tricetin (3′,4′,5′,7′-pentahydroxylavone)</td>
<td>5.00</td>
<td>0.00885</td>
<td>1780</td>
</tr>
<tr>
<td>3,4-Dihydroxybenzoic acid</td>
<td>215</td>
<td>0.00802</td>
<td>37</td>
</tr>
</tbody>
</table>

FIGURE 5. Reaction schemes comparing product formation with 3,4,5-trihydroxycinnamic acid as substrate. A, SynOMT from Synechocystis sp. strain PCC6803 (SynOMT) and B, PFOMT from M. crystallinum. S, substrate 3,4,5-trihydroxycinnamic acid; P1, 3,5-dihydroxy-4-methoxycinnamic acid; P2, sinapic acid; P3, 5-hydroxy-3,4-dimethoxycinnamic acid; P4, 5-hydroxyferulic acid. In the case of SynOMT, P4, the precursor for P2 and P3, could never be detected by HPLC and is therefore illustrated in reduced intensity.

The values in parentheses represent the values for the highest resolution shell.

* The values in parentheses represent the values for the highest resolution shell.

** The root mean square deviation for bonds and angles are the rmd from ideal values.

contact with the catalytic magnesium atom, with the methyl group juxtaposing the AdoHcy thiol group and in the neighborhood of absolutely conserved Lys<sup>146</sup>, which could act as a catalytic base for a nucleophilic methyl transfer reaction as suggested for rat COMT (11). The meta-substituted ferulic acid, on the other hand, demonstrates an unproductive binding mode, with the para-hydroxy group directed toward the cofactor thiol and the meta-methylated function pointing away from the metal ion. No specific stabilizing interactions for this latter binding mode are observed; a corresponding arrangement of feruloyl-CoA was also observed for CCoAOMT (10).

Previously unobserved in the plant CCoAOMTs, the N-terminal residues of SynOMT reach into the active site, with the amino group of Lys<sup>3</sup> closely approaching the magnesium ion and the meta-hydroxyl group of isoferulic acid.
As small rearrangements of the side chain could allow direct participation in the catalytic reaction, Lys³ was mutated to alanine. The resultant enzyme showed no activity toward caffeic acid conjugates or trihydroxylated substrates. A very low activity could be determined only in the case of 5-hydroxyferulic acid and caffeic acid (0.7 and 0.6% as compared with the wild type protein) upon prolonged incubation times, indicating that Lys³ is absolutely required for enzymatic activity with hydroxylated substrates.

To identify structural features of unknown in vivo substrates of SynOMT, two databases were screened for compounds consistent with the docking arrangement of the ligands in the x-ray structure of neighboring phenolic hydroxyl groups complexed by a magnesium allowing for a catalytic methylation of one hydroxyl group via AdoMet (Table 5). From more than 1000 putative ligands in the MOE and KEGG databases about 100 showed higher affinities than caffeic acid and ferulic acid. Best scores were obtained for plant-derived hydrolysable tannins laevigatin D and pentagalloylgucose (31, 32) both with several aromatic trihydroxy groups (Table 5). A search for putative substrates in Synecochyrtis-related prokaryotes revealed the antimitotic cryptophycins, 16-membered macrolides from the cyanobacteria Nostoc (33). Interestingly, cryptophycin 1 contains an aromatic methoxy group in the para-position and a chlorine in the meta-position. The compound showed a higher in silico affinity for the active site of SynOMT than the enzymatically tested hydroxycinnamic acid derivatives (Table 5).

DISCUSSION

SynOMT from Synechocystis sp. strain PCC 6803 is the first cation- and AdoMet-dependent O-methyltransferase to be characterized and crystallized from a prokaryote. Although the natural substrate of this enzyme is currently unknown, the substrate specificity for vicinal dihydroxy groups of phenolics is comparable with the currently known eukaryotic proteins. The position specificity is strikingly different, with promiscuity toward both meta- and para-positions never observed for any of the eukaryotic enzymes. A comparable specificity has been described for the prokaryotic SafC gene product from M. xanthus when tested with caffeic acid, although the natural substrate for the latter enzyme appears to be 1-dihydroxyphenyllalanine, a biosynthetic precursor of the isoquinoline alkaloid saframycin (13). The precise structural features of the Synecochyrtis protein that contribute to this unexpected phenomenon are as yet unclear. One possibility is provided by side chains of His¹⁷⁴ and Lys¹⁷⁶ in the neighborhood of the propenoic acid moiety, equivalent to Asn and Ser in the plant CCoAOMTs, respectively (supplemental Fig. S1). Curiously, two distinct binding modes for the monomethylated caffeic acid products, ferulic acid and isoferic acid, were found. Whereas the positioning of the aromatic ring oxygen atoms of isoferic acid suggest a substrate-like interaction with the active site magnesium and the AdoMet cofactor, ferulic acid binds with only one oxygen approaching the metal ion.

Although the N-terminal lysine, Lys³, must be involved in the catalytic mechanism, the almost complete abrogation of activity on mutation to alanine suggests a more fundamental role than determining position specificity. N-terminal residues have already been implicated in position specificity determination in the plant enzymes (34). Loss of two N-terminal lysine residues in the plant enzyme PFOMT results in a sequential and partial loss of activity (2, 10, 12), whereas the presence of an N-terminal His tag influences substrate specificity (12). The individual contribution of N-terminal lysine residues in the plant enzymes...
Interestingly, the dimeric structure of the OMT of minor differences in position specificity could reside here. N-terminal regions of each of these enzymes, suggesting that minor differences in position specificity could reside here. Interestingly, the dimeric structure of the OMT of L. interro-gans (14), in which the N termini are domain-swapped, suggests that the N-terminal residues could switch from the active site of one monomer to the other, allowing for a wider variety of potential products as observed for PFOMT and SynOMT. The monomeric cation-dependent mammalian COMTs lack any lysine residues in the N-terminal region (5).

In contrast to hydroxycinnamic acids, flavonoids with para-methylated B-rings are observed throughout the plant kingdom (35), although methylation is sometimes performed by cation-independent enzymes with strict structural requirements. For example, a flavonoid-specific OMT from Catharanthus roseus methylates the 4’-position only when the neighboring 3’-methoxy group of the flavonoid B-ring is present, as in the case of the isorhamnetin or homoeriodictyol (36). Isohamnetin is not a substrate of SynOMT, as it lacks the required vicinal dihydroxy system. A similar cation-independent OMT, cloned from rice and functionally expressed in E. coli, performs sequential methylation of three B-ring hydroxyl groups of the flavone tricetin in vitro, including the para-position (37). In contrast to the plant proteins, the para-position is an initial target of the prokaryotic enzyme, at least in the case of the phenylpropanoid esters, followed by the subsequent attack of either the 3’-OH or 5’-OH group. With trihydroxylated flavonoids, tricetin and myricetin, these subsequent methylation reactions are missing and only a single product is observed with SynOMT.

Although 3,4,5-trihydroxycinnamic acid has so far not been found to occur in the plant kingdom, 5-hydroxyferulic acid, the aldehyde, alcohol, or the corresponding caffeic acid derivatives could provide potential in vivo substrates for this new type of enzyme. It is probably not that surprising that para-methylated intermediates of lignin biosynthesis are unknown. Current lignin models favor radical-radical rather than enzyme-mediated coupling with an unsubstituted para-hydroxy group required for resonance stabilization to promote cross-linking of different monomer units (38). Para-methylated (oligo) lignins would destabilize radical formation resulting in reduced cross-linking and a different lignin structure. An enzymatic activity such as that described here for SynOMT could therefore be detrimental to lignin formation. Tremendous efforts have been undertaken to manipulate the composition of lignin in various plant species with the focus on methyllating enzymes (7, 39, 40) or those involved in earlier biosynthetic steps, e.g. cinnamyl alcohol dehydrogenase and cinnamoyl-CoA reductase (41). Expression of enzymes targeting para-hydroxy groups in the appropriate gymnosperm or angiosperm background may allow for the production of novel types of lignin with altered properties such as changes in the degree or mode of polymerization that might affect digestibility by ruminants (42).

With the set of substrates tested in this system, the metabolic function of this enzyme in cyanobacteria remains unclear. The conserved presence of at least one gene locus in most prokaryotes points to an important in vivo function for these orga-nisms including Synechocystis. Sequence identities to the SafC gene product from M. xanthus methylating the saframycin precursor 1-dihydroxyphenylalanine (13) as well as to the \( \text{mdmC} \) gene in Streptomyces mycarfaciens, proposed to encode the 4-O-methyltransferase for the 16-membered lactone ring of the macrolide antibiotic midecamycin (43) point to a possible role in the biosynthesis of complex secondary metabolites. In contrast to the bisquinone structure of saframycin, the midecamycin molecule gives no indication of any aromatic vicinal dihydrox system, therefore the proposed methylation step remains questionable. SynOMT is also not part of any recognizable gene cluster as is the case of S. mycarfaciens and the closest neighbors do not give any hint to its possible function (Fig. 1). A role in methylation of a macrolide or an aromatic precursor of a quinoid metabolite is plausible. Macrolides like the cryptophycins from Nostoc sp. (34) may also be present in Synechocystis and could provide an initial basis for detailed metabolite profiling analyses.

The essential function of the \( \text{slr0095} \) gene product might be further resolved by knock-out mutations and homologous recombination, feeding of labeled precursors, and molecular docking studies. Cloning and functional expression of other members of the prokaryotic CCaOMT-like proteins may hold further surprises as far as substrate and position specificities toward natural compounds are concerned and the enzymes might be used as specific organic catalysts in yet difficult to achieve chemical modifications.

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O-Methyltransferase from Synechocystis

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Functional and Structural Characterization of a Cation-dependent \( O \)-Methyltransferase from the Cyanobacterium \textit{Synechocystis} sp. Strain PCC 6803

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