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A single amino acid determines position specificity of an *Arabidopsis thaliana* CCoAOMT-like *O*-methyltransferase

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ABSTRACT

Caffeoyl-coenzyme A *O*-methyltransferase (CCoAOMT)-like proteins from plants display a conserved position specificity towards the *meta*-position of aromatic vicinal dihydroxy groups, consistent with the methylation pattern observed in vivo. A CCoAOMT-like enzyme identified from *Arabidopsis thaliana* encoded by the gene *At4g26220* shows a strong preference for methylating the *para* position of flavanones and dihydroflavonols, whereas flavones and flavonols are methylated in the *meta*-position. Sequence alignments and homology modelling identified several unique amino acids compared to motifs of other CCoAOMT-like enzymes. Mutation of a single glycine, G46 towards a tyrosine was sufficient for a reversal of the unusual *para*- back to *meta*-*O*-methylation of flavanones and dihydroflavonols.

1. Introduction

Methylation performed by *S*-adenosyl-L-methionine (AdoMet)-dependent *O*-methyltransferases (OMTs) (EC 2.1.1) is a common alkylation reaction observed in the biosynthesis of natural products. It modulates the chemical reactivity and renders the physiological properties of aromatic compounds more hydrophobic [1]. In plants, OMTs with a preference for caffeoyl coenzyme A esters designate a group of Mg^{2+}-dependent and low molecular weight (23–27 kDa) enzymes (CCoAOMTs). They play an important role in the methylation of guaiacyl lignin subunits in gymno- and angiosperms, which are crucial for the structural integrity of this important polymer [2]. Substrate-tolerant or promiscuous CCoAOMT-like enzymes were identified from several plant species which in addition to caffeoyl CoA methylate a variety of metabolites with catechol-type functionality, including various types of flavonoids, anthocyanins, coumarins, and aromatic esters [3]. The position specificity however, is always conserved and restricted towards methylation of the *meta* hydroxyl group in aromatic catechols. This is consistent with the metabolite pattern observed in vivo, resulting in vanilloid types of substitution patterns [4,5].

CCoAOMT-like enzymes involved in an isovanilloid type of methylation pattern (*para*-*O*-methylation) have been described from cyanobacteria [6] but are not known from plants. A *para* or 4-*O*-methylation activity is observed from another clade of plant OMTs, the cation-independent enzymes. One example is described from *Catharanthus roseus*[7]. This enzyme displays promiscuous position specificity in several catechol-like substructures of flavonoids although the in vivo substrate is not known. In case of the cation-independent isoeugenol OMT from fairy fan (*Clarkia breweri*) evolutionary *plastic* amino acids E165 and T133 among various others resulted in catalytically efficient variants capable of 4-*O*-methylation of some lignin monomers, like coniferyl- and sinapoylalcohol. These mutated enzymes were used to alter and repress lignin monomer biosynthesis in the cruciferous plant *Arabidopsis thaliana*[8].

In this report we describe an endogenous member of the CCoAOMT-like enzyme family from *A. thaliana* encoded by the gene *At4g26220* which in vitro displays unusual 4-*O*-methylation specificities for flavanones and dihydroflavonols resulting in an isovanilloid type of methylation pattern. Sequence alignments and computational predictions led to the identification of a single amino acid responsible for this unique functionality. As a reference enzyme for modelling and sequence comparison, the phenylpropanoid and flavonoid OMT from *Mesembryanthemum crystallinum* (PFOMT) was used (Ibdah et al., 2003). The data are discussed with respect to structural aspects, enzyme design, and in vivo function of the newly discovered activity.

2. Materials and methods

2.1. Materials

Eriodictyol, hesperetin and homoeriodictyol (racemic mixtures) as well as taxifolin (2*R*,3*R*) were obtained from Roth (Karlsruhe,

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Germany). Several flavonoids and hydroxycinnamic acids were from Extrasynthese (Genay, France) or are from our in house compound collection. Methylated flavanone and dihydroflavanone mono and dimethyl ethers were also provided by Eckard Wollenweber (Darmstadt, Germany).

2.2. Cloning, expression, and purification

Heterologously expressed OMTs cloned into expression vector pQE30 (Qiagen, Hilden, Germany) were grown in the *Escherichia coli* strain M15 (Qiagen) containing the plasmid pRep4 and expression was induced with 1.0 mM isopropyl-thio-β-D-galactoside (IPTG). Two liters of crude bacterial extract were prepared by centrifugation (10000×*g*, 4 °C, 15 min) and subsequent ultrasonication of the resultant pellet according to published protocols [3]. Preparation of purified enzymes was performed by affinity chromatography on 1 ml HiTrap HP (Amersham, Freiburg, Germany) as published previously [9]. Total yields varied from 1 to 10 mg of pure CCoAOMT-like proteins/L depending on the enzyme. All purifications were monitored by SDS–PAGE. Enzyme concentrations were calculated based on the corresponding extinction coefficients at 280 nm. Purified enzymes were stable for months when stored at −80 °C. Whereas the recombinant PFOMT [3] was also stable at 4 °C, CCoAOMT7 encoded by *At4g26220* lost 50% activity when stored over night at 4 °C. Mutagenized constructs of *At4g26220* were synthesized by Geneart (Regensburg, Germany) without codon optimization and cloned into p-MA-T (Geneart) using the corresponding restriction sites compatible for cloning directly into pQE30 (Qiagen) for functional expression in M15pRep4 cells and subsequent purification.

2.3. Enzyme assays

Standard enzyme assays were conducted at 37 °C for 60 s up to 300 s in 10 mM KPi buffer, pH 7.5 in a total volume of 50 µl containing a final concentrations of 10% (v/v) glycerol, 250 µM $MgCl_2$, and 5% (v/v) DMSO with varying concentrations of 2–40 µM of the individual methyl group acceptor (dissolved in 40% (v/v) DMSO), 300 µM AdoMet and 1 µg of purified enzyme. The reactions were terminated by addition of 20 µl 5% TCA in 50% (v/v) acetonitrile. After removal of precipitated protein by centrifugation (2 min, 18000×*g*) the reaction products were analyzed by reversed phase liquid chromatography on a Nucleosil 5 µm C_{18} column (25 cm × 4 mm i.d.; Macherey and Nagel, Düren, Germany) with a linear gradient from 20% to 70% (v/v) acetonitrile (solvent B) in 0.5% (v/v) aqueous phosphoric acid (solvent A) within 12 min at a flow rate of 1 ml min^{-1}. Detection of substrates and products was performed either at 310 nm (eriodictyol and taxifolin) or at 345 nm (luteolin). The K_m and V_{max} values for eriodictyol and luteolin were obtained after adjusting assay conditions for each substrate individually using at least six individual substrate concentrations and six individual time points each. Data were visualized by Lineweaver–Burk plots and quantified by linear regression analysis. All enzyme activities and kinetic data were recorded in triplicates, based on two independently performed experiments.

2.4. Sequence alignment, molecular modelling, and docking

Sequence alignments were performed using the Lasergene software package (DNASTAR, Madison, WI, USA). The X-RAY structure of PFOMT, deposited in the pdb-database (3C3Y) appeared as a best template [6] with a sequence identity of 64%. Based on this alignment, a dimeric 3D-structure of CCoAOMT7 was modelled with the molecular modelling MOE [10] allowing outgap modelling of the seven N-terminal residues. The model was refined with the md-refinement option in YASARA [11]. The quality of the model was checked with PROSA II [12] and PROCHECK [13]. Equatorial and axial conformations of eriodictyol were optimized with DFT B3LYP 6-31G+(d,p) basis set to determine the energy differences between both conformations using GAUSSIAN 03 [14]. Since in case of eriodictyol the axial conformation was calculated to be only 0.3 kcal/mol less stable than the corresponding equatorial conformation, all four basic structures (*R*- and *S*-eriodictyol, each with equatorial and axial conformation, were considered for docking studies. Fifty docking runs were performed for eriodictyol and luteolin using GOLD [15,16] with the ChemScore fitness function and for all other options with standard settings. For the definition of the active site, the magnesium ion in PFOMT, in wild type and in mutants of CCoAOMT7 was chosen as origin with a binding region of 15 Å. The following amino acid side chains were set to be flexible applying the rotamer library included in GOLD: in CCoAOMT7 wild type: D4, K7, Q44, M47, R194; in case of the CCoAOMT7 G46Y mutant additionally Y49, and for the quadruple mutant M194 instead of R194; and in case of PFOMT the corresponding amino acids. The docking results were analysed manually using MOE considering the conformations which were in a reactive positions, i.e. at a close distance (<3.4 Å) between a phenolic oxygen to the methyl carbon atom of AdoMet to be transferred.

3. Results and discussion

3.1. cDNA cloning, functional expression, and purification

Several cation-dependent CCoAOMTs from *A. thaliana* and from various plant species only synthesize *meta* or 3′-*O*-methylated hydroxycinnamic acids and flavonoids [3]. When the recombinant CCoAOMT-like enzyme encoded by the *A. thaliana* gene *At4g26220*, termed CCoAOMT7 [17] was cloned and functionally expressed, methylation of the standards caffeic acid and caffeoyl CoA resulted in ferulic acid and feruloyl CoA formation, as expected (Table 1). In contrast, an unusual methylation pattern with the flavanone, eriodictyol was observed. Instead of a single methylation product, two product peaks were detected in a constant 80:20 ratio (Figs. 1 and 2). The major product was identified as hesperetin or eriodictyol 4′-*O*-methylether by comparison with an authentic standard, whereas the minor one co-eluted with a standard of eriodictyol 3′-*O*-methylether (homoeriodictyol). Exclusively homoeriodictyol was produced by all other CCoAOMTs tested during this screen (data not shown) consistent with initial expectations. No dimethylated compound was ever observed even after prolonged incubation times. In case of caffeicacid, the flavone luteolin or the flavonol quercetin, only a single *meta*-methylated product was synthesized by CCoAOMT7.

To investigate this unusual positional promiscuity of CCoAOMT7 in case of eriodictyol in more detail, sequence alignments were performed with several CCoAOMT-like enzymes used

Table 1
Relative activities of recombinant CCoAOMT7 encoded by the gene *At4g26220* towards selected methyl group acceptors. 100% activity is equivalent to 4.0 nkat/mg recombinant and purified protein in case of luteolin (average of triplicates). Aromatic substrates with no vicinal hydroxyl groups like kaempferol or scopoletin were not accepted.

Compound	Relative activity
Luteolin	100
Quercetin	60
CaffeoylCoA	33
Esculetin	20
Eriodictyol	15
Caffeic acid	10
Dihydroquercetin	2.5