Regiospecificity and kinetic properties of a plant natural product *O*-methyltransferase are determined by its N-terminal domain

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Abstract A recently discovered, *S*-adenosyl-L-methionine and bivalent cation-dependent *O*-methyltransferase from the ice plant, *Mesembryanthemum crystallinum*, is involved in the methylation of various flavonoid and phenylpropanoid conjugates. Differences in regiospecificity as well as altered kinetic properties of the recombinant as compared to the native plant *O*-methyltransferase can be attributed to differences in the N-terminal part of the protein. Upon cleavage of the first 11 amino acids, the recombinant protein displays essentially the same substrate specificity as observed earlier for the native plant enzyme. Product formation of the newly designed, truncated recombinant enzyme is consistent with light-induced accumulation of methylated flavonoid conjugates in the ice plant. Therefore, substrate affinity and regiospecificity of an *O*-methyltransferase in vivo and in vitro can be controlled by cleavage of an N-terminal domain.

Key words: Methyltransferase; N-terminal domain; Regiospecificity; Phenylpropanoid; Flavonoid

1. Introduction

S-Adenosyl-L-methionine (SAM) and Mg^{2+}-dependent class I *O*-methyltransferases (OMTs) are present throughout the animal and plant kingdom. In animals they are referred to as catechol *O*-methyltransferases (COMTs), involved in the inactivation of neurotransmitters or detoxification of mutagenic phenolics [1,2]. In plants they are usually associated with methylation of a single substrate, caffeoyl coenzyme A (CCoA), a key intermediate in lignin biosynthesis [3,4]. Novel Mg^{2+}-dependent enzymes have been described for the ice plant, *Mesembryanthemum crystallinum*, for chickweed, *Stellaria longipes*, and also for Arabidopsis [5]. These enzymes, unlike the specific enzymes involved in lignin formation, show a very tolerant substrate specificity towards a variety of flavonoids, phenylpropanoids, and phenylpropanoid esters, like caffeoyl glucose and CCoA. Despite their tolerance in substrate specificity, both subclasses display the same absolute regiospecificity towards aromatic vicinal dihydroxy groups, comparable to their animal counterparts [2].

Cloning and expression of the phenylpropanoid and flavonoid *O*-methyltransferase (PFOMT) from the ice plant indicated a remarkable difference in regiospecificity of the recombinant PFOMT versus the native plant protein. Instead of three products obtained by the native enzyme with quercetagetin, five different products were observed for the recombinant enzyme (Fig. 1). In addition to quercetagetin (queg)-3′-*O*-methylether (OMe), queg-6-OMe, and queg-6,3′-diOMe, two highly fluorescent 5-*O*-methylated quercetagetin derivatives, the 5-OMe and the 5,3′-diOMe were identified [5]. However, in the ice plant, upon irradiation with high light intensities, only 6- and 3′-methylated products accumulate as complex glycosylated flavonol conjugates, consistent with the activity of the native plant enzyme [5–7]. The easily detectable, fluorescent compounds could not be observed in light irradiated plants. In this report, we demonstrate that the differences in product formation can be attributed to the differences in the N-terminal domain between the native enzyme [5], and the newly designed, recombinant full-length and N-terminally truncated versions of the PFOMT described in this report.

2. Materials and methods

Based on the sequence of the PFOMT cDNA, primers N-0 (5′-CGGGATCC*ATG*GAT-TTTGCTGTGATG-3′), N-5 (CGGGATCC*ATG*AAGCAGGTCAAAAATA), N-11 (5′-CGGGATCC*ATG*ACAGGATTGCAG-3′) were designed corresponding to the N-terminal target sequences, with *Bam*HI restriction site (GGATCC) to facilitate cloning into the vector pQE32 (Qiagen, Hilden, Germany). The codon for the N-terminal Met is shown in italics. Likewise, the reverse primer (CTCAAGCTTGTGTCAATAAAGACGCCTGCAG), targeted to the C-terminus, contained a *Hin*dIII restriction site (AAGCTT). cDNAs were obtained by polymerase chain reaction (PCR) amplification of the PFOMT cDNA, by a proof reading polymerase, pfu (Promega, Mannheim, Germany). Individual amplified fragments were separated on agarose gels, bands cut and eluted from the gel by glass milk (Qiagen), digested with *Bam*HI and *Hin*dIII to remove adaptors, ligated into pQE32 vector, transformed into M15 [rep4] cells (Qiagen), and selected on ampicillin according to standard procedures. cDNAs were verified by sequencing on a Licor (MWG Biotech, Ebersberg, Germany). For initial screening, 3 ml from eight individual clones were picked, grown in Luria–Bertani (LB) amp^+ medium, induced with 1 mM isopropyl β-thiogalactoside (IPTG) at an optical density $(OD)_{600nm}$ of 0.6, grown for additional 4 h at 37°C, and lysed by ultrasonication in Buffer A (100 mM KPi, pH 7.0; 10% glycerol, 1 mM 2-mercaptoethanol). The supernatant was directly assayed for enzyme activity by high-performance liquid chromatography (HPLC) as described [5]. All extracts were also subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). The clones with the highest specific activities were selected for preparative protein purification.

6 l each of IPTG-induced *Escherichia coli* cells containing the different PFOMT cDNAs grown at 37°C for 4 h, were centrifuged for 5 min at $10\,000 \times g$, the pellet ultrasonicated in 200 ml of Buffer A, recentrifuged and stirred with 0.05% protamine sulfate for 5 min to

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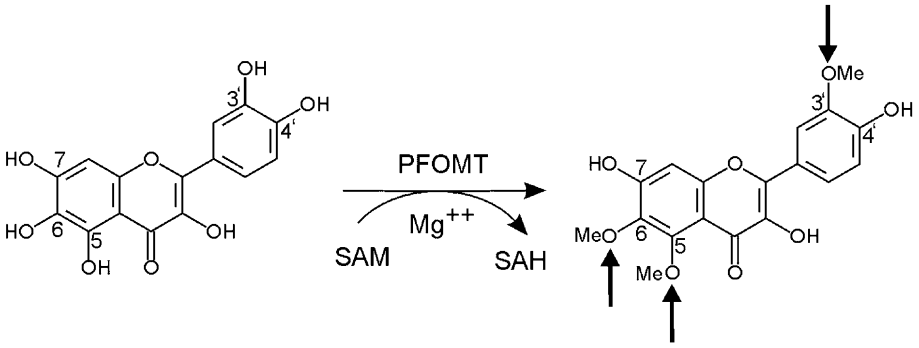

Fig. 1. Reaction scheme of the methylation of the major endogenous flavonol aglycone, quercetagetin from light irradiated ice plants. Arrows indicate all possible methylation sites by the recombinant PFOMT proteins.

precipitate the nucleic acids. The extract was centrifuged and the supernatant directly applied to 25 ml of TALON affinity matrix (Clontech, Palo, Alto, CA, USA) equilibrated in Buffer A, at a flow rate of 3 ml/min. The column was washed with 10 and 30 mM imidazole pH 7.0, subsequently, and the active protein eluted with 240 mM imidazole, pH 7.0 in a total volume of 30 ml. Protein concentrations were quantified by the absorbance at 280 nm with a calculated extinction coefficient of 18 730 ± 5% and were calculated between 1.6 and 2.0 mg/ml. Individual proteins were assayed for activity and stored at −80°C until further determination of kinetic properties. Proteins were checked for purity by SDS–PAGE.

Kinetic properties of the enzymes were determined from two independent experiments each performed in triplicate with five different substrates, at six different substrate concentrations from 2 to 40 μM (in case of SAM from 10 to 400 μM). 1–10 μg of purified enzymes were used per assay. Assays were analyzed by reversed phase HPLC chromatography [5]. K_m and K_{cat} data were calculated based on Lineweaver–Burk plots and linear regression analysis. Quercetagetin, quercetin and caffeic acid were purchased from Extrasynthese (Genay, France). Caffeoyl glucose and CCoA were prepared as described [5].

3. Results

N-terminal sequencing of the native plant protein started with amino acid 12 of the protein sequence, a threonine. The deduced amino acid sequence from the full-length clone obtained by screening a cDNA library, indicated that the first 11 amino acids were missing [5]. Although this is not uncommon for plant enzymes, it is usually characteristic for proteins with signalling sequences [8]. However, there is no known signal sequence in the N-terminus of PFOMT as indicated by the program Signal P [9].

To check whether the observed differences in regiospecificity towards the endogenous quercetagetin could be attributed to the observed differences in the N-terminal domain or to the additional His-tag associated with the recombinant protein, three different proteins were designed and expressed, each with an N-terminal His-tag to facilitate identification and purification (Fig. 2). The reference protein (termed N-0) contained the full-length amino acid sequence. From the second protein, termed N-5, the first five amino acids were deleted. From the third sequence (N-11) the first 11 amino acids were deleted. This protein corresponded to the native plant enzyme, as N-terminally sequenced upon purification [5]. An N-terminal methionine was added to this sequence to initiate possible translation without a His-tag, since some degradation of the His-tagged recombinant protein was observed in earlier experiments [5]. Expression of all recombinant isoforms in *E. coli* and purification after protamine sulfate treatment followed by affinity purification yielded high amounts of essentially pure, soluble, and catalytically active protein in all three cases, with protein yields ranging from 8 (N-0) to 10 mg of apparently pure protein (N-5 and N-11) per l *E. coli* culture (Fig. 2). In contrast to the initial purification [5], no degradation products or other contaminating protein sequences were observed. This is due to a change in purification strategy, but also to the elimination of contaminating vector sequences [5]. The HPLC pattern, which illustrates position specificity towards the endogenous plant substrate quercetagetin is shown for all three different proteins in Fig. 3. With the full-length protein five different products were detected, including the two fluorescent derivatives, compounds 1 and 3 observed earlier [5]. The product profile of the novel truncated proteins is different. This difference is already obvious with the truncated N-5 protein. Although there is no apparent difference in product quality and the fluorescent compounds are still observed, the pattern is already shifting towards a more rapid methylation of quercetagetin at positions 6 and 3′ resulting in a rapid formation of queg-6,3′-diOMe (compound 6). The latter compound is only slowly formed by the full-length PFOMT sequence, although it is the predominant flavonol detected in light irradiated ice plant leaves [7]. The product pattern of N-11 is drastically different. Only two major products are formed, queg-6-OMe (peak 5) and the 6,3′-diOMe derivative (peak 6). No traces of any fluorescent 5-OMe derivatives have ever been detected with this enzyme. From these data, it can

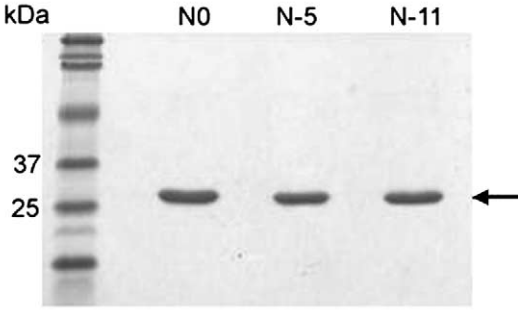

Fig. 2. 10 μg of affinity-purified, recombinant PFOMT proteins separated on a 14% SDS–PAGE gel. M, molecular weight markers; N-0, *MGHHHHHHGS*MDFAVMKQVKN**TGLLQSEILCQYIL**..., 28.0 kDa full-length clone; N-5, *MGHHHHHHGS*MKQVKN-**TGLLQSEILCQYIL**..., 27.4 kDa, missing five amino acids from the N-amino-terminal; N-11, *MGHHHHHHGS***MTGLLQSEILC-QYIL**..., 26.8 kDa, missing 11 amino acids from the N-amino-terminal. The His-tags are marked in italics, the sequenced N-terminal part of the native PFOMT is written in bold letters. The arrow marks the position of the purified enzymes.

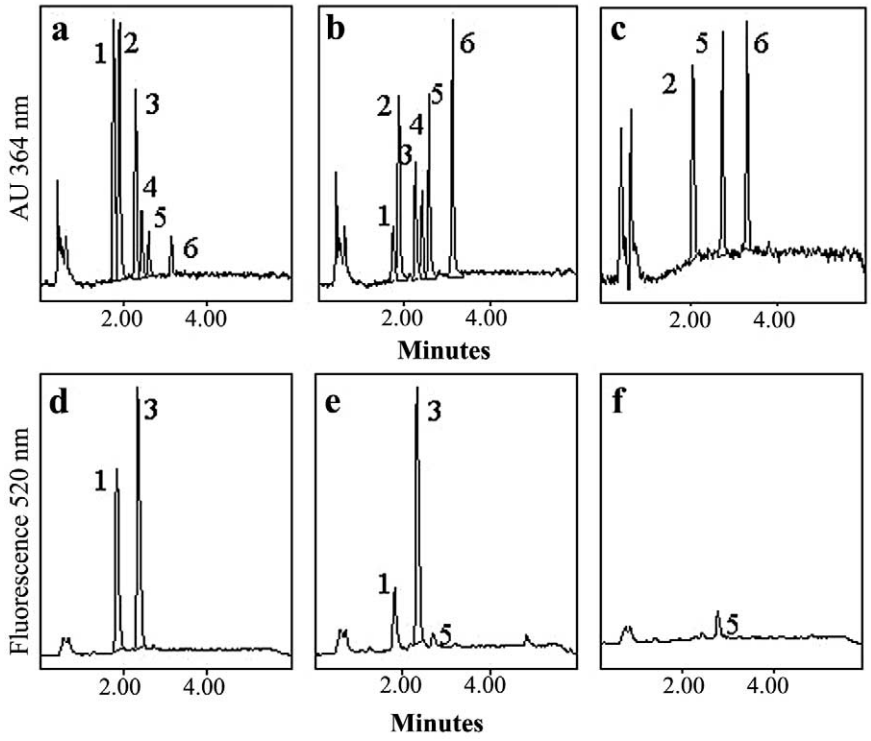

Fig. 3. Analysis of reaction products of recombinant PFOMT isoforms. N-0 (a,d), N-5 (b,e), and N-11 (c,f) with quercetagetin as the substrate by HPLC. Ultraviolet (UV) detection at 364 nm (a–c) and fluorescence detection (d,e), excitation at 370 nm and emission at 520 nm. Peak identification: peak 1, queg-5-OMe; peak 2, queg (substrate); peak 3 queg-5,3′-diOMe; peak 4, queg-3′-OMe; peak 5, queg-6-OMe; peak 6, queg-6,3′-diOMe. Only Peaks 2, 4, 5, 6 are also observed with the native enzyme. Fluorescing peaks 1 and 3 are products of N-0 and N-5 recombinant enzymes only.

be concluded that the native protein, described in an earlier report [5] and this recombinant protein in vitro display a comparable specificity for the endogenous substrate quercetagetin. Pattern formation is not dependent on the presence of a His-tag and does also not depend on pH (tested between pH 5 and 9). Affinities towards SAM are unaffected by the N-terminal truncations, and K_m values between 30 and 35 μM were calculated (data not shown).

Kinetic data were calculated with five different substrates and are illustrated in Table 1. Consistent with previous data, the full-length N-0 has the highest affinity to caffeoyl glucose, which appears to be the best in vitro substrate [5]. Inhibition of the full-length N-0 with the flavonoids already occurred at a concentration as low as 12 μM, therefore kinetic measurements with quercetagetin and quercetin were performed between 1 and 10 μM. This inhibitory effect was never observed with caffeic acid and caffeic acid esters up to concentrations of 40 μM. The data indicate that the full-length enzyme is comparable to the N-5 version in kinetic properties. This enzyme also showed less pronounced inhibition by quercetagetin and quercetin and concentrations up to 20 μM could be used. Cleavage of the 11 amino acids from the PFOMT results in a drastic reduction in catalytic efficiency. PFOMT N-11 displayed lower catalytic efficiencies in vitro for all substrates analyzed. This is especially obvious in case of caffeoyl glucose, where a dramatic reduction in K_{cat} is observed between the N-5 and the N-11 PFOMT. This is not due to a reduced affinity, but is correlated with a significant reduction in K_{cat} only. Although the catalytic efficiency dropped also for the endogenous plant substrate quercetagetin, it is the best substrate for this recombinant enzyme in vitro. The recombinant plant enzymes display a similar affinity and comparable, although slightly lower, turnover rates than the corresponding class I animal COMTs [2,10]. Comparable catalytic efficiencies and

Table 1
Apparent in vitro kinetic properties of purified, recombinant PFOMT proteins

PFOMT substrate	N-0			N-5			N-11		
	K_m (μM)	K_{cat} (s^{-1})	K_{cat}/K_m (s^{-1} M^{-1})	K_m (μM)	K_{cat} (s^{-1})	K_{cat}/K_m (s^{-1} M^{-1})	K_m (μM)	K_{cat} (s^{-1})	K_{cat}/K_m (s^{-1} M^{-1})
Quercetagetin	30	0.27	9000	17	0.14	8200	39	0.052	1300
Quercetin	25	0.11	4400	12	0.089	7400	11	0.0043	470
CCoA	49	0.089	1800	50	0.067	1300	99	0.0027	27
Caffeoyl glucose	7.1	0.067	9400	31	0.14	4500	49	0.00081	16
Caffeic acid	21	0.041	1900	42	0.051	1200	23	0.0062	270

1–40 μM of individual substrates were incubated with 1–10 μg of purified recombinant proteins at 30°C. The data represent the average of two independent measurements. Substrate and products were separated by reversed phase HPLC [5].

relatively low in vitro turnover rates have been described for the recently crystallized plant natural product class II OMTs [11], which are not dependent on bivalent cations.

4. Discussion

Our results demonstrate that regiospecificity, substrate affinity, and catalytic efficiency of a member of plant natural product Mg^{2+}-dependent *O*-methyltransferases are determined by the N-terminal domain. From our recently published enzyme purification assays with the native plant protein, the N-terminally truncated sequence was initially regarded as a purification artefact [5]. From the new data obtained with the recombinant enzymes, it is now plausible that product formation by the N-terminally truncated PFOMT, either recombinant or native, is consistent with the observed product profile in the ice plant. The extreme reduction in catalytic efficiency of the truncated N-11 protein with selected substrates (caffeoyl glucose) may reflect yet unknown aspects of metabolic regulation, and should merit further investigations towards localization of the PFOMT. A similar interest may be directed towards a (specific?) endogenous protease, cleaving the native plant protein probably in statu nascendi to result in the observed product pattern and specificity. If similar mechanisms exist in other plants, like *S. longipes* or Arabidopsis remains to be investigated.

We cannot exclude that additional mechanisms exist in the ice plant, which modify or regulate the in vivo activity and specificity of PFOMT. The N-terminal domain contains three highly significant phosphorylation sites, Ser^{32}, Ser^{50}, and Ser^{54}, as determined by the NetPhos 2 software package [12]. But, as known from a variety of examples, these phosphorylation sites may likely be involved in the regulation of protein activation and signalling cascades rather than determination of substrate specificity [13–15]. Based on significant yields of recombinant His-tagged proteins from *E. coli*, we have initiated crystallization studies to solve the structure of this newly discovered enzyme. X-ray data should reveal the essential amino acids involved in determination of regiospecificity in molecular detail.

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