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RESEARCH ARTICLES

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Partial characterization and photolabeling of a *Rhizobium meliloti* polysaccharide methyltransferase with S-adenosylmethionine

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Summary S-Adenosylmethionine (SAM) has been used to directly cross-link a polysaccharide specific methyltransferase isolated from *Rhizobium meliloti* HA. This peculiar enzyme transfers a methyl group to the 2-O-galacturonosyl residue of a teichuronic type polysaccharide and was very unstable. The apparent K_m for SAM was 0.46 mM. The Hill coefficient, n, was 1. The enzyme had an optimum pH of 8.2 and requires Mn^{2+} at concentration of 2 mM. The enzyme was inactivated by saline concentrations of 120 mM or greater and was eluted from Superose columns with an apparent molecular weight of 28 kDa. The isoelectric point was close to 7.0. To elucidate the relationship between chemical structure and catalytic function, (^{3}H)SAM was cross-linked to the enzyme and the enzymatic activity was assayed in presence and in absence of commercial substrate analogs. Cross-linking was performed by direct irradiation of enzyme and (^{3}H)SAM. The uptake of radioactivity was linear up to about 20 min and then reached a plateau. This irreversible junction is specific, as shown by a number of different criteria. Several competitive inhibitors were able to affect this photoactivated cross-linkage. As the concentration of inhibitors increased, both, the level of photolabeling and enzyme activity always decreased. The SAM-enzyme adduct was shown to be a single protein band by SDS polyacrylamide gel electrophoresis.

Key words *Rhizobium meliloti* · S-Adenosylmethionine (SAM) · 2-O-Methyltransferases · UV irradiation · Enzyme inhibitors

Introduction

Irreversible covalent binding of an enzyme to its substrate by UV irradiation has been used to identify the binding site. S-Adenosylmethionine (SAM) participates in a number of enzymatic reactions involving the transfer of methyl groups [4]. These methyltransferases include enzymes that result methylate proteins, nucleic acids, lipids, various small molecules and less commonly, oligosaccharides [6, 11]. The use of (^{3}H)SAM as a photolabel probe was first reported by Yu who demonstrated a covalent linkage between (^{3}H)SAM and phenylethanolamine N-methyltransferase [8, 28]. The unmodified SAM has been shown to photolabel other SAM-dependent enzymes: myo-inositol 6-O-methyltransferase [23], protein carboxyl methyltransferase, *Eco*RII methyltransferase [15], Dam methylase [25] and the key enzyme in caffeine biosynthesis [11]. The first report of mammalian methyltransferase in covalent adduct with SAM under UV light demonstrated the involvement of a tyrosine residue [17]. An identical observation was described for *Escherichia coli* DAM methyltransferase [26].

A large number of prokaryotic and eukaryotic methyltransferases and other SAM-utilizing enzymes are distinguished by the presence of three sequence motifs, that may be related to the binding of their common substrate. The cDNA of the L-isoaspartyl methyltransferase gene from the nematode *Caenorhabditis elegans* was obtained utilizing degenerate oligonucleotide primers derived from conserved aminoacid sequences of mammalian, plant and bacterial enzymes and PCR amplification [9].

Others studies were carried out using 8-azido-S-adenosylmethionine as photoprobe in specific photolabeling but this compound is more unstable than (^{3}H)SAM [10].

Previous observations showed that a polysaccharide of *Rhizobium meliloti*, was associated with infectivity and cell surface changes, modifying the interaction with antisera, alfalfa agglutinin and phages [7]. The teichuronic structure of the cell surface

polysaccharide was demonstrated and the sensitivity to phage 16B was associated with its presence [18, 19]. Two variants were isolated from laboratory stock cultures of *R. meliloti* strain 102F51. One was highly agglutinable (HA) by an alfalfa seed agglutinin, sensitive to phage F20 and resistant to phage 16B and the other showed low agglutinability (LA), resistance to F20 and sensitive to phage 16B. Mutants selected by phage resistance, lacked the enzymes required for the transfer of galactose from UDP galactose and/or galacturonic acid-insoluble inner membrane endogenous acceptor. Subsequently, Coira et al [5] described a soluble enzyme that catalyzed the transfer of methyl groups from S-adenosylmethionine to position 2 of the galacturonosyl residue of teichuronic acid. In the present article, data are presented demonstrating that teichuronic methyltransferase can be photolabeled with SAM. A detailed investigation was carried out studying simultaneously the kinetics and the effect of analogs upon photolabeling and enzymatic activity.

Materials and methods

Materials 2-O-Methyltransferase was partially purified from the mutant strain 102 F51 of *Rhizobium meliloti* HA. This mutant was obtained as previously reported [7]. All SAM analogs were purchased from Sigma. L-, D- and LD-methionine were purchased from Aldrich. Adenosyl-L-methionine (S[methyl-^{3}H]) was purchased from NEN Research Products, with 73.8 Ci/mmol of specific activity and a concentration of 0.0030 mg/ml. Other chemicals were of the highest purity available from commercial sources and were used without further purification.

Preparation of methyl acceptor *R. meliloti* 102F51 LA was grown in liquid yeast-extract medium [21]. The inner membrane was obtained as previously reported [5]. This preparation, adjusted to an equivalent glucose content of 1 mg/ml, was used as acceptor in enzymatic activity determinations.

Methyltransferase assay Enzymatic activity was assayed in 20 mM Tris-HCl pH 8.2, 2 mM $MnCl_2$, 0.5 mCi (^{3}H) methyl-S-adenosyl-L-methionine and 10 mg of glucose equivalents of methyl acceptor as previously reported [5]. Background radioactivity was estimated from samples without the addition of protein.

Radiolabeling of methyltransferase with (^{3}H) SAM Adenosyl-L-methionine (S-[methyl- ^{3}H]) was incubated with protein samples in the same buffer used for the activity determination without methyl acceptor at a final volume of 100 µl. The mixture was placed in round bottom 96 well microtiter plates and the cross-linking of (^{3}H)SAM and methyltransferase was induced by irradiating with 254 nm UV light source at 4°C. The reactions were stopped by adding a 10% sodium dodecyl sulfate containing 30 mM Tris (pH 8.0), 27% sucrose, 1% Nonidet P-40, 6% 2-mercaptoethanol and 3 mM EDTA. The cross-linking was determined from the amount of radioactivity precipitable by 10% TCA. Photolabeled samples were analyzed by 12% SDS gel electrophoresis and soaked in 10 volumes of 1 M sodium salicylate (pH 6.0) for 30 min and exposed to X-ray film with an enhancing screen at –80°C. Previous to radiolabeling, the radiochemical purity was checked by paper chromatography.

Partial purification of methyltransferase *R. meliloti* HA was grown in liquid yeast extract medium at 28°C and harvested at mid-log phase. The cells were resuspended in 30 mM Tris-HCl pH 8.2, 10 mM EDTA and 20% sucrose and treated with lysozyme (150 mg/ml) at 4°C for 1 hour. The cells were harvested by centrifugation, redissolved in 25 mM Tris-HCl pH 9.0, 10 mM EDTA, 1 mM dithiothreitol (DTT), 10% glycerol and 5 mM CHAPS (Buffer A). Raw material was prepared from the 100,000 × *g* overnight supernatant of cells disintegrated with French Press. 1 mM phenylmethylsulfonyl fluoride (PMFS) was used. The supernatant was precipitated with acid or ammonium sulfate (30–70%). The acid precipitation was performed by adding formic acid until pH 4,5. The precipitate was recovered by centrifugation and resuspended in different buffers.

We performed a test tube method to roughly determine the pI value of the enzyme. The addition to each tube of known constant amounts of sample (in buffer A) and the assay of enzymatic activity allowed us to determine the optimal pH to perform DEAE batch adsorption.

The preparations obtained were concentrated with ultrafiltration and fractionated on a Sephadex G-75 superfine (4.8 × 60 cm) equilibrated in buffer A. The fractions with enzymatic activity were concentrated by another ultrafiltration and used for K_m and K_i determinations and photolabeling. All steps described above were performed in the same day.

When the acid precipitate was recovered in 25 mM Bis-Tris-Propane pH 9.0, 10 mM EDTA, 1 mM DTT, 10% glycerol and 5 mM CHAPS (Buffer B) the sample was fractionated on a Mono Q HR 5/5 anion exchange column equilibrated with the same buffer. The loaded column was washed isocratically followed by a linear gradient (40 ml) from 0 up to 100% of the same buffer but pH 7.0 (Buffer C).

Alternatively, the methyltransferase samples were resuspended in 50 mM Tris-HCl pH 8.2, 1 mM EDTA, 1 mM DTT, 5% glycerol and 5 mM CHAPS/CHAPSO (Buffer D) and were applied on a Superose 12 and Superose 6 HR 10/30 MW. The detection was carried out by measuring enzymatic activity in fractions collected every 30 s at a flow rate of 0.25 ml/min.

Finally, buffer D was used to recover the protein previous to the loading of a S-adenosyl-L-homocysteine agarose matrix (Bethesda Research Laboratories-BRL). The bound protein was eluted in buffer D with 5 mM of SAM (Buffer E).

Preparative flat-bed electrofocusing A preparative electrofocusing in granulated gel was performed. We used wide pH gradients (3.5–9.5) and the measurements were performed directly with a surface electrode. The running conditions were: 16 W constant power at 10°C overnight. When separation was completed the gel bed was sectioned using 20 blades in a fractionating grid frame. We transferred each section to elution columns using a spatula and resuspended the gel in 75 mM

Tris-HCl pH 8.2; 0.1 mM EDTA, 1 mM DTT and glycerol 10% (Buffer G). Enzymatic activity was determined in 25 µl aliquots of each eluate.

Chromatofocusing The chromatofocusing separations were performed on MONO P HR 5/20 (Pharmacia) at broad pH intervals. The buffer systems were 0.075 M Tris-CH_3COOH pH 9.3/ Polybuffer 96-CH_3COOH pH 6.0; 0.050 M Bis-Tris-propane pH 8.0/Polybuffer 74 pH 5.0 and 0.025 M Bis-Tris pH 7.0/Polybuffer 74 pH 5.0 as starting and eluting buffers respectively.

Protein determination Protein concentration was determined by the method of Bradford [3].

K_m and K_i determinations For K_m determination of 2-O-methyltransferase, the activity was assayed varying the concentrations of (^{3}H)SAM and SAM (0.25, 0.5, 1, 2, 2.5, 5, 10, 15 and 20 mM). The analogs were added to the incubation mixture without preincubation in concentrations from 0.5 up 250 mM. Slope replots were used to obtain true inhibition constants [14].

Capillary electrophoresis separations Capillary electrophoresis (CE) was performed by using a Waters Quanta 4000 Capillary Electrophoresis System as previously reported [12].

Results

Partial purification and stability of 2-O-methyltransferase The enzyme was partially purified and the specific activities obtained are presented in Table 1. The kinetic experiments were performed with an activity mean of 18,474 units/mg protein. The purer methyltransferase samples were very unstable at room temperature, 4°C, and –80°C. Addition of DTT and glycerol seems to protect the enzyme to some extent. The DEAE supernatant, retained more than 95% of its original activity for more than 2 months when kept at –80°C. The apparent K_m for S-adenosylmethionine was 0.46 mM and the Hill coefficient was 1. The enzyme had a narrow pH range with an optimum at 8.2 and it required Mn^{2+} at 2 mM final concentration.

We observed that at 0.5 mM, p-chloromercuribenzoate decreased enzymatic activity to 20 %; inhibition being almost parallel to the increase of SH reagent concentration (data not shown). Surprisingly, the enzyme also was inactivated by saline concentrations of 120 mM or greater. This inactivation is not reversible by dialysis or dilution. Attempts to stabilize the enzymatic activity by adding analogs, albumin, and polyethylene glycol (PEG) MW 8000 were unsuccessful. Moreover, we studied the influence of biological detergents on the methyltransferase activity. Only cationic detergents (the same charge of SAM in acid pH) inhibited enzyme activity. Under the same conditions, zwitterionic molecules were activators. Unfortunately, the addition of detergents did not prevent the inhibition by freezing and thawing.

The enzyme eluted from Superose 6 and Superose 12 columns with an apparent molecular weight of 28 kDa in buffer B. In Mono Q the enzymatic activity was eluted in pH 7.5. The preparative isoelectrofocusing in flat-bed showed that the pK_i is located between 7.0–7.4. By chromatofocusing the values oscillated between 6.8–7.4.

We used affinity chromatography repetitively to obtain enough amounts of enzyme for the MW_{app} determination by SDS-PAGE. This preparation was homogeneous and revealed identical MW to one determined by molecular

Table 1 Purification of the *Rhizobium meliloti* HA methyltransferase[a]

Material	Units/ml	Volume ml	Total units $\times 10^3$	Yield %	Protein mg/ml	Specific activity (units/mg protein)
Crude extract	10842	40	433.68	100	3.82	2830
Acid precipitacion		19732	20	394.64	91	4.21 4680
Ammonium sulfate (30–70)	13785	20	275.70	64	2.86	4820
Supernatant DEAE	14103	20	282.06	65	2.73	5170
Sephadex G-75	7066	20	141.32	33	0.42	16824
Ultrafiltrated	68722	2	137.32	32	3.72	18474
Methyltransferase samples obtained directly from DEAE supernatants						
Superose 12	6892	2	13.78	3.17	0.97	7110
Mono Q	2408	1	2.40	0.55	0.24	10040
Isoelectric focusing	11780	4	47.12	11	1.31	8992
Chromato-focusing	1579	2	3.16	0.73	0.22	7177

[a] Units = 1000 cpm of (^{3}H) methyl 5% trichloroacetic acid-insoluble × min^{-1}

filtration under non-denaturant conditions. In our conditions, the affinity sample did not have activity and resolved as a faint band in monodimensional gel when Coomasie blue staining was used (Fig. 1).

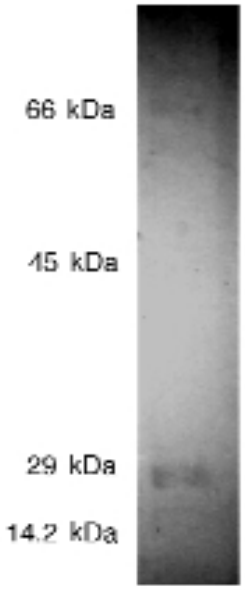

Fig. 1 SDS-PAGE of 2-O-methyltransferase sample obtained by affinity chromatography

Effect of UV irradiation on 2-O-methyltransferase activity in presence and absence of (^{3}H)SAM The enzyme preparation was irradiated in absence of SAM. At different times, aliquots were withdrawn and the remnant methyltransferase activity was determined. We observed that the UV light causes inactivation presumably through a first-order photochemical reaction. In another experiment, (^{3}H)SAM was irradiated with the same UV light used in cross-linking reactions for 20 min at 4°C. Immediately after this irradiation, methyltransferase samples were added and further incubated for 20 min. No radioactive labeling was detected. This evidence indicated that both, enzyme and substrate, must be in close contact during the formation of the radiolabeled adduct.

The maximal incorporation of ^{3}H was observed after 20 min and reached a plateau (Fig. 2). No radioactivity was fixed in the absence of UV light and preincubation was found unnecessary. The crosslinking yield was estimated to be 0.0078 +/– 0.0027% by ratio between cpm added to radioactivity incorporated. The addition of DTT did not affect the photoreaction. We also observed that the presence of Mn^{2+} is necessary for the photolabeling and the dilution of partially purified samples led to an increase of incorporated radioactivity. Under the same conditions, bovine serum albumin, cythocrome C and the heat-treated (90°C, 2 min) enzyme did not show photolabeling (data no shown).

We performed a partial purification of SAM-enzyme cross-linked adduct by gel filtration. The adduct eluted at fractions where a molecular weight of 28 kDa was estimated. Alternatively, we used a fast desalting column. In both procedures the untreated enzyme (as determined by measuring the enzyme activity) coincided with radiolabeled protein-SAM complex (data not shown). This adduct was analyzed by SDS-electrophoresis revealing a single protein-band by fluorography. Boiling of samples for 3 min prior to loading them onto SDS-gel did not affect the intensity of the band observed.

Effect of SAM analogs on the in vitro 2-O-methyltransferase activity and on the cross-linking reaction Several structural analogs were assayed as effectors on the activity of 2-O-methyltransferase, including aromatic and aliphatic compounds that mimic parts of the entire S-adenosylmethionine molecule. At 0.25 mM analog concentration all compounds tested were inhibitors with the exception of methionine. S-Adenosyl-homocysteines, homocystines and S-adenosyl-L-cysteine showed maximal inhibition activity. Under the same conditions, DL-methionine-S-methyl methylsulfonium chloride, 5'-deoxy-5'-methyl-

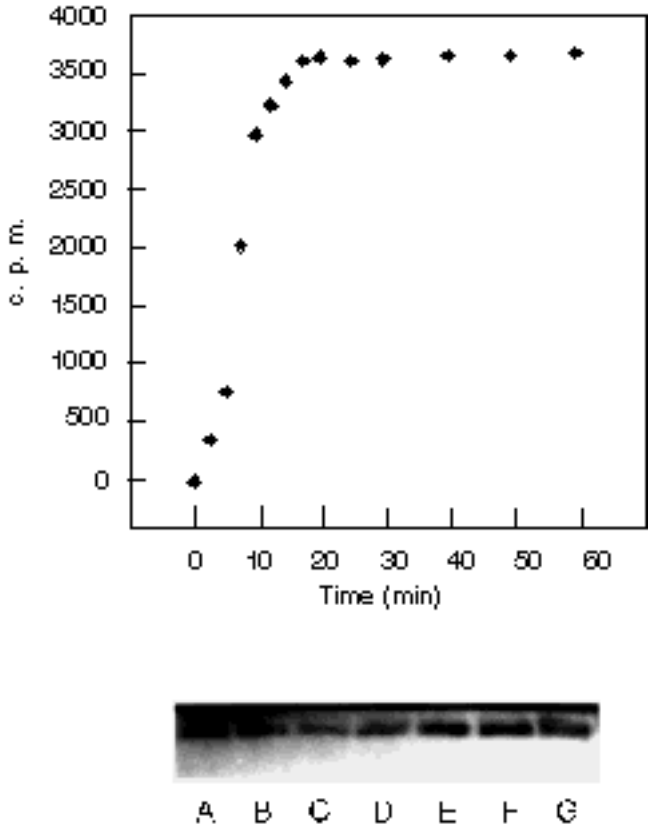

Fig. 2 Time course of UV-induced radioactivity incorporation from (^{3}H)SAM. Aliquots were withdrawn at: (a) 2.5, (b) 5.0, (c) 10, (d) 15, (e) 30, (f) 40, and (g) 60 min, and analyzed by fluorography as described under Materials and methods

tioadenosyne, L-cysteine and DL-homocysteine inhibited 42%, 77%, 53% and 84% respectively. At lower concentrations D- and L-S-adenosyl-homocysteines showed as the strongest inhibitors. All analogs were found to be competitive (Table 2). It is interesting to note that the $K_{m\ app}$ of the methyltransferase for SAM was found to be higher than K_i of its product in both optical isomerical conformations. Similar results were observed when D- and L-homocystines were used.

Table 2 Inhibitor constants of SAM analogs for 2-O-methyltransferase[a]

Inhibitor	K_i (mM)
D-S-Adenosyl-homocysteine	0.07
L-S-Adenosyl-homocysteine	0.08
D-Homocysteine	0.27
L-Homocysteine	0.26
S-Adenosyl-L-cysteine	0.38
DL-Methionine-S-methyl methylsulfonium chloride	2.85
5'-Deoxy-5'-methylsulfonium chloride	0.80
DL-Cysteine	2.14
DL-Homocysteine	0.77

[a] The K_i constants were calculated from the data obtained by replotting on the $K_{m\ app}$ vs. inhibitor concentrations.

The analysis by capillary electrophoresis of SAM revealed the appearance of a new peak at major t_m. These observations were not reproducible when radioactive SAM was injected in the same conditions. Stock by stock, the intensity of the additional peak oscillated between 14 to 18% of total and was assigned to unnatural diasteroisomer [12].

For photolabeling, the different protein samples were exposed over 20 min in the presence of 5 mCi (^{3}H)SAM in an ice bath. Adduct formation was dramatically diminished when no-radiolabeled SAM was added and 0.5 mM was sufficient to abolish it. Further experiments demonstrated that the analogs assayed could also prevent the photochemical cross-linking and this inhibition in the photolabeling is proportionally for its concentration and K_i's values. Such competition was not observed when ATP or methionine were assayed (data not shown).

Discussion

Only about 3% of the 3196 enzymes described in the latest version of *Enzyme Nomenclature* [24] are proteins that catalyze the attack of a variety of nitrogen, oxygen, carbon and sulfur nucleophiles on the methyl group of S-adenosylmethionine. These enzymes are involved in many biochemical reactions that are crucial for biological processes. Determination of multiple alignments revealed three regions of sequence similar in SAM dependent-methyltransferases and non-methyltransferases. The motifs are always found in the same order and have comparable intervals [20]. However, other groups of methylases, which also employ SAM, do not contain the sequence motifs conserved in DNA methylases, for instance, CheR methylase [16]. This enzyme has two cysteine residues essential for enzyme activity but differing to 2-O-methyltransferase of this study, the DTT inhibited the photolabeling. Cysteine was shown to be a key residue for catalysis in all of the DNA cytosine 5-methyltransferases studied so far. Our results agree with the observations of Hurts [8] for phenylethanolamine N-methyltranferase, suggesting that free radical formation is not sufficient to explain adduct formation.

In the crosslinking experiment, radioactively labeled SAM could be replaced by competitive inhibitors including S-adenosyl homocysteine. These observations may show competition for catalytic center of methyltransferases but, as it has been published there are two different sites of SAM binding. These inhibitors can compete with SAM for the binding to the catalytic site and not for the allosteric one [1, 27]. It is interesting to remark that the inhibitory effects of S-adenosyl-homocysteines are more potent than the inhibition caused by other analogs. These are common observations in methyltransferases but not for *Eco*RI methyltransferase. Also, we noticed that both optical isomers of S-adenosylhomocysteine were good inhibitors of the 2-O-methyltransferase enzyme activity and photolabeling; these observations are different from those reported by Som and Friedman for *Eco*RII methyltransferase [15]. However, the K_i S-adenosylhomocysteine/K_m SAM ratio ranges widely for different SAM-dependent enzymes from 0.133 to 200 [25]. In our conditions the ratio was close to 150. The structural basis for the variation in sensitivity to product inhibition shown by these enzymes are not understood but the presence of an unnatural diasteroisomer of SAM in commercial preparations is very important because this may determine an increase in $K_{m\ app}$ value and a decrease of K_i values of analogs.

A viable strategy to facilitate the purification of these enzymes may rely on chemically stable and inactive SAM analogs. The absence of the sulfonium center enhances the chemical stability of analogs, for instance, sinefungin [2, 13, 22]. Possibly the analogs studied here may be helpful in studies about other methyltransferases.

Finally, we considered that this newly characterized enzyme would provide an useful model for the study of structure-function relationships.

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