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Molecular characterization of the C-methyltransferase NovO of *Streptomyces spheroides*, a valuable enzyme for performing Friedel–Crafts alkylation

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ABSTRACT

The methyltransferase NovO cloned from *Streptomyces spheroides* could be heterologously produced as soluble and active enzyme in *Escherichia coli*. Sequencing of the cloned *novO* gene revealed differences to the GenBank entry AAF67508.1 resulting in a different amino acid at position 223 (Cys instead of Ser). A generated variant containing a Ser residue at this position, however, resulted in poor ability to express soluble and enzymatically active protein. Characterization of NovO revealed a type I methyltransferase that performs its action as a dimer in solution. Functional elements include the conserved S-adenosyl-L-methionine (SAM) binding site (consensus: E/DXXXGXG) as DLCCGSG (residues 45–51). Mutation analyses of the respective amino acids verified their importance for cofactor binding and enzyme activity. In soluble protein fractions of mutants D45N and G49A the calculated k_{cat} values decreased from $2.5 \times 10^{-2}\ s^{-1}$ of the wild-type protein to $9.7 \times 10^{-4}\ s^{-1}$ and $1.2 \times 10^{-3}\ s^{-1}$, respectively. A histidine at position 15 was identified as the catalytic base in the methyl transfer reaction. The analysis of purified enzyme preparations showed that the transfer of allyl groups via the SAM analog allyl-SAH occurs with a fourfold increased k_{cat} of $11 \times 10^{-3}\ s^{-1}$ compared to $3.2 \times 10^{-3}\ s^{-1}$ for methyl transfer. However, the evolutionary design toward SAM is obvious from the K_m value of 0.06 mM compared to 0.22 mM for allyl-SAH.

1. Introduction

Methylation is one of the key reactions in all living organisms. Methyl groups cause steric and electronic effects in small molecules and on biomolecules, which lead to consequences in biological behavior such as selectivity among receptors, activity and protection strategies [1]. The transfer of methyl groups is catalyzed by methyltransferases. These enzymes need cofactors such as tetrahydrofolic acid, betaine and vitamin B12 but the majority of these enzymes use S-adenosyl-L-methionine (SAM) as methyl donor, which is the second most widely used cofactor in nature [2]. The SAM-dependent methyltransferases act on different substrates including DNA, RNA, proteins, lipids, carbohydrates and small molecules. The enzymes are highly specific for the atom they methylate. The methyl group can be transferred to S—, N—, O—, C— and even halogen atoms [3]. The methyl transfer from SAM to its substrate is associated with very favorable enthalpy ($\Delta H \approx -70$ kJ/mol) and leads to selective methylation [4]. Due to their versatile substrates the role of the enzymes is diverse, including for example functions such as regulation, signaling, storage and biosynthesis. Several SAM dependent C-methyltransferases (sterol-C-, RNA- and DNA-nucleoside-C-methyltransferases) are known [5–7]. Apart from methyltransferases involved in the biosynthesis of polyketides [8], ubiquinone and its analogs [9] until now only few C-methyltransferases, which act on small aromatic substrates, were identified [10–12]. In nature the production of antibiotics very often depends on the methylation of precursors. A special group of these antibiotics, which are produced by *Streptomyces* species, exhibits potent antimicrobial activity by targeting DNA gyrase [13]. Coumermycin, clorobiocin, and novobiocin belong to this family of coumarin antibiotics. Novobiocin (Albamycin; Pharmacia & Upjohn) has been licensed in the United States as an antibiotic for the treatment of infections with multiresistant Gram-positive bacteria such as *Staphylococcus epidermidis*

Abbreviations: SAM, S-adenosyl-L-methionine; SAH, S-adenosyl-L-homocysteine; coumarinbenzamide, *N*-(4,7-dihydroxy-2-oxo-2*H*-chromen-3-yl) benzamide.

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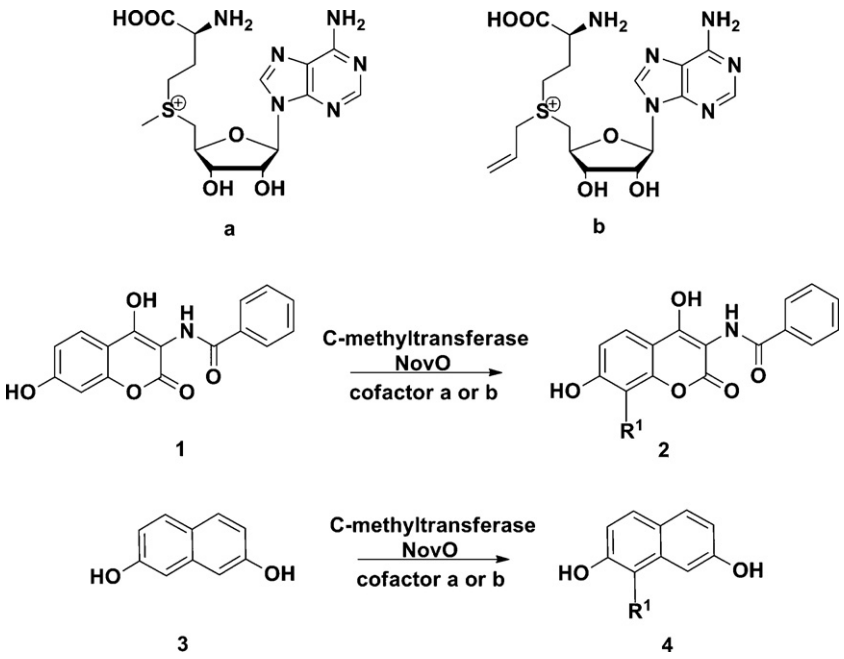

Scheme 1. Alkyltransfer catalyzed by NovO illustrated with cofactors SAM **a** and allyl-SAH **b**. SAM **a** was purchased and allyl-SAH **b** synthesized as described previously (22). R^1 = methyl, allyl. **1**: *N*-(4,7-dihydroxy-2-oxo-2*H*-chromen-3-yl) benzamide, **2**: *N*-(4,7-dihydroxy-8-alkyl-2-oxo-2*H*-chromen-3-yl) benzamide, **3**: 2,7-dihydroxynaphthalene and **4**: 1-alkyl-2,7-dihydroxynaphthalene.

and *Staphylococcus aureus* [14–16]. In the last decade great effort has been made to unravel the biosynthesis of these antibiotics, leading to an understanding of the involved enzymes and the order of their action [17–26]. Concerning the biosynthesis of novobiocin it has been shown that NovO is the methyltransferase that is required for methylation of desmethylnovobiocic acid [22]. Recently, we reported on a novel enzymatic C—C bond formation reaction, namely the Friedel–Crafts alkylation catalyzed by SAM-dependent methyltransferases (Scheme 1). The substrate acceptance of these enzymes was broader than expected and more surprisingly, the enzymes also accepted chemically modified cofactors as alkyl donors. Thus, alkylation of aromatic substrates was not restricted to methylation [27].

Here we present a detailed molecular and biocatalytic characterization of the SAM-dependent methyltransferase NovO in order to rationalize its role and mode of action in the enzymatic Friedel–Crafts alkylation.

2. Experimental

2.1. Cloning, heterologous expression and purification

The methyltransferase NovO of *Streptomyces spheroides* (DSMZ 40292) was cloned, heterologously expressed and purified as described previously [27], with the exception that no inducer was added to recombinant *Escherichia coli* cultures. Two alternative primer pairs were used to check the correctness of the amplified *novO* gene (Table S1 #25–28). NovO variants were generated by site-directed mutagenesis using the appropriate primers listed in Table S1 [28]. Lysis of pET26b(+)-novO-variants, and pET26b(+)-novO-Strep-variants was achieved by incubation in 20 ml of 50 mM sodium phosphate buffer of pH 6.5 with 6 mg lysozyme (Roth, Karlsruhe, Germany) per gram wet cell paste at room temperature for 20 min and subsequent incubation with 250 U Benzonase® (Merck, Darmstadt, Germany) per gram wet cell paste at room temperature for another 20 min. Aliquots of total crude extracts were analyzed on SDS-PAGE. The resultant cell debris was removed by centrifugation (60 min at 50,000 × *g*). The supernatant was analyzed by SDS-PAGE and kept at 4 °C for enzymatic reactions and further affinity chromatography. Purified fractions were pooled and concentrated with ultra-filtration spin columns (cut-off 10,000 Da) from Vivaspin (Sartorius, Aubagne, France) to a final concentration of 5–10 mg/ml. For storage at −20 °C glycerol was added to a final concentration of 50%. Total protein concentrations were determined by Bradford assay (BioRad, Hercules, USA) using bovine serum albumin (Thermo Fisher, Waltham, USA) as reference protein. Quantitation of respective protein bands in SDS-PAGE was done with the GeneTools software from SynGene® (Syngene, Cambrigde, UK).

2.2. Methyltransferase assays for activity determination toward substrate analogs

Soluble protein fractions of recombinant *E. coli* expressing NovO or its variants were incubated in 0.1 ml scale in a thermomixer at 35 °C and 1000 rpm for 16 h. The reactions contained 0.5 mM coumarinbenzamide **1** from a 10 mM stock solution prepared in DMSO, or 2,7-dihydroxynaphthalene **3** from a 40 mM stock solution prepared in 10% of DMSO, 90% of 50 mM sodium phosphate buffer pH 6.5, and 2 mM SAM **a** (Sigma–Aldrich, St. Louis, USA) from a 20 mM stock solution prepared in 50 mM sodium phosphate buffer pH 6.5, and 0.1 mg/ml BSA from a 2 mg/ml stock solution prepared in 50 mM sodium phosphate buffer of pH 6.5. The amount of soluble protein fractions was set to 80% of the total reaction volume. Following incubation at 35 °C reactions were stopped by heating at 80 °C for 10 min and centrifuged at 16,000 × *g* for 15 min. 10 μl of the aqueous solution was analyzed by HPLC. For determination of relative activities the reactions were incubated for 16 h and for determination of specific activities of soluble fractions the mixtures were incubated for 5, 10, 20, 40, 80, and 160 min. Specific activities were calculated based on determining the content of NovO in the soluble protein fractions by gel quantitation. All reactions were done in triplicate.