Pergamon

Bioorganic & Medicinal Chemistry Letters 9 (1999) 1533–1536

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

ISOLATION AND CHARACTERIZATION OF AN ACTIVE-SITE PEPTIDE FROM A STEROL METHYL TRANSFERASE WITH A MECHANISM-BASED INHIBITOR

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Received 4 March 1999; accepted 21 April 1999

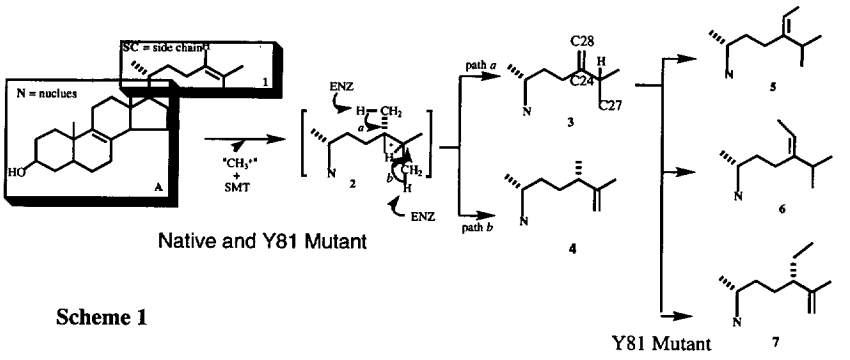
Abstract: Chemical affinity labeling of pure sterol methyl transferase (SMT) from *Saccharomyces cerevisiae* using the mechanism-based irreversible inhibitor, [3-^{3}H]26,27-dehydrozymosterol, inhibited the SMT with an apparent K_i of 1.1 μM and k_{inact} of 1.52 min^{-1}. The protein-inhibitor adduct was subjected to cleavage with trypsin and the resulting covalently modified peptide was analyzed by Edman sequencing from the N-terminus. The radiochemically labeled ca. 5.0 kDa peptide fragment of the cleavage mixture was shown to be contiguous through 17 residues to a segment that includes a highly conserved hydrophobic motif (Region I, stretching between T78 and F91) characteristic of SMT enzymes. The results confirm that Region I is the sterol binding/active site.

Sterol methyl transferases are a family of *C*-methyl transfer enzymes that catalyze the conversion of olefinic precursors to a variety of 24-methyl and 24-ethyl sterol side chains. SMT enzymes are ubiquitously represented in plants, fungi and marine organisms. Together these enzymes are capable of converting the universal sterol acceptor molecule with a Δ^{24}-bond into more than 200 distinct phytosterols (sterols with a 24-alkyl group in the side chain).[1] The molecular diversity in product structure and stereochemistry, is to a great extent, controlled by the topology of the active site and alternate binding orientations of the $\Delta^{24(25)}$- and $\Delta^{24(28)}$-sterol substrates, viz., the steric-electric plug model.[2] The *C*-methylation pathways of phytosterol synthesis have been established.[3] Less-advanced plants (algae and fungi) synthesize primarily 24β-methyl sterols whereas advanced-plants (vascular plants) synthesize 24α-ethyl sterols, suggesting that the size and stereochemistry of the 24-alkyl group is functionally and phylogenetically significant. Very little is known, however, about the way SMT enzymes catalyze, control and evolve *C*-methylation pathways to mono and doubly *C*-methylated side chains or the nature of the SMT active site.

The $\Delta^{24(25)}$- to $\Delta^{24(28)}$-*C*-methylation enzyme synthesized by *Saccharomyces cerevisiae* operates the C_1-transfer reaction only and converts zymosterol **A1** to fecosterol **A2** stereoselectively by the same mechanism catalyzed in plants (path a, Scheme 1).[3] The native yeast SMT, a tetramer of 172 kDa, has been cloned and overexpressed in *Escherichia coli*.[4] Recent evidence from site-directed mutagenesis experiments indicate the involvement of a hydrophobic motif that is unique to SMT enzymes.[5] This motif, flanked by a set of highly conserved amino acid residues T78 and F91 in the yeast SMT (referred to as Region I; Figure 1) plays a role in sterol binding and catalysis.[4] Thus, when tyrosine-81 is replaced with phenylalanine zymosterol is converted to fecosterol; in addition, three different 24-ethyl sterol products are generated from a $\Delta^{24(28)}$-sterol substrate, including 24β-ethyl cholesta-8,25(27)-dienol (side chain **7**). These mechanistic results resolve the long-standing

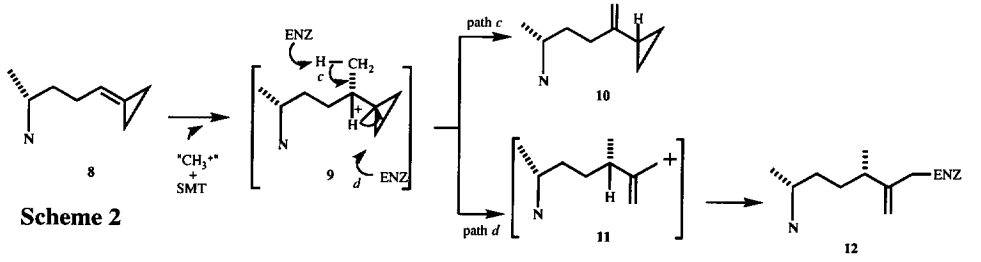
0960-894X/99/$ - see front matter
PII: S0960-894X(99)00242-5

controversy whether the first C_1-transfer and second C_1-transfer reactions can be catalyzed by a single enzyme from the same binding site using the same *Si*-face (β-face) methylation mechanism.[3 - 5]



Scheme 1

To pursue our analysis of SMT enzymology further, we recently reported the synthesis of a rationally designed, mechanism-based irreversible inactivator based on the structure of the preferred substrate for the yeast SMT, 26,27-dehydrozymosterol (DHZ) **A8**,[6] which made it possible to determine the stoichiometry of binding and covalently modify the active site of the enzyme. The mechanism of inhibition is proposed to involve initial C-methylation of carbon-24 in the side chain of 26,27-DHZ to the allylic C26 cation, which can be trapped by an active-site nucleophile, resulting in irreversible inhibition of the SMT (**12**, Scheme 2). Alternatively, turnover of the inhibitor with side chain **8** can proceed to **10**, as demonstrated in cycloaudenol biosynthesis (side chain 4).[7] We describe herein the chemical affinity labeling of the pure SMT from yeast using [3-^{3}H]26,27-DHZ with an apparent kill to turnover ratio of 1.0.



Scheme 2

Incubation of increasing concentrations of [3-^{3}H]26,27-DHZ with pure recombinant SMT enzyme (0.42 μM) and [^{3}H-*methyl*]AdoMet (100 μM; 20.0 mCi/mmol) at 30 ^{0}C in Buffer A (50 mM TrisHCl, pH 7.5, $MgCl_2$ 2 mM β-mercaptoethanol, 20 % glycerol) failed to generate a *C*-methylated sterol product in the absence of zymosterol, but in the presence of zymosterol gave rise to pseudo-first-order time-dependent inactivation of the SMT enzyme activity, as evidenced by the linear dependence of the log of residual activity against time (Figure 2). The rate of inactivation by 26,27-DHZ was saturable, with a maximum rate of inactivation, k_{inact} of 1.52 ± 0.01 min^{-1} ($t_{1/2}$ = 2.10 min) at 0.83 μM protein and a K_i for 26,27-DHZ of 1.1 ± 0.01 μM. Co-incubation of 5 μM 26,27-DHZ with 50 μM and 100 μM **A1** with zymosterol afforded protection against inactivation

generating 44% and 65% *C*-methylation activity, respectively, relative to the *C*-methylation activity of a control incubation that contained saturating amounts of substrate and coenzyme only.

Figure 1. Alignment of amino acids from several plant and fungal SMT enzymes corresponding to Region I. Refer to reference 8 for a key to **A** to **K**; Top sequence contains the tryptic digest fragment Arg62 to His107.

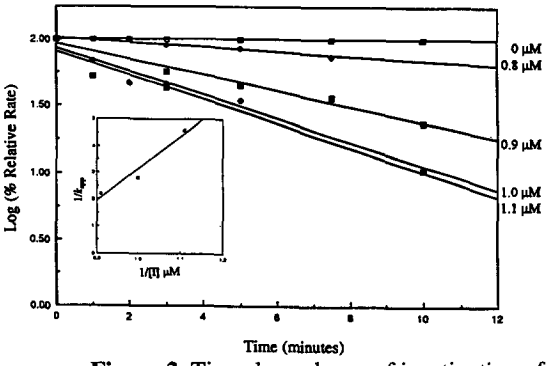


Figure 2. Time dependency of inactivation of *S. cerevisiae* SMT enzyme by **A8**. Four experiments were performed and the variation among the trials did not exceed 5%.

The covalent nature of binding was established by incubation of 100 µM [3-^{3}H]26,27-DHZ with 0.83 µM SMT enzyme in Buffer A for 45 min. After dialysis and Centricon (Y30) filtration to concentrate the protein and remove unbound inhibitor, the protein sample was boiled in SDS buffer for 10 min prior to analysis by chromatgraphic separation on 12% SDS-PAGE gels. Staining with Coomassie blue confirmed the presence of a single protein of the expected M_r, while treatment of the gel with DMSO-2,5-diphenyloxazole and radiofluorography revealed the presence of a single radioactive band with mobility identical to that of authentic SMT enzyme (Figure 3). In control experiments, [3-^{3}H]26,27-DHZ was separately incubated with boiled SMT enzyme. Analysis of the corresponding SDS-PAGE gels, either by radiofluorography or by excision of the relevant protein bands and direct liquid scintillation counting failed to indicate any comigration of tritium with protein, thereby confirming the specificity of the covalent modification of SMT enzyme by 26,27-DHZ. Tryptic digest of the SMT-[3-^{3}H]inhibtior adduct followed by chromatographic separation of the sample on 20% SDS-PAGE gels demonstrated that a single band was labeled correpsonding in M_r to ca. 5 kDa (Figure 4). The adduct, electroeluted from the gel and Edman sequenced (17 residues), was found to possess a peptide fragment which started at Arg62 and contained Region I (Figure 1). These results, together with the site-directed mutagenesis experiments involving Y81 show that Region I is the sterol binding/active site.

To determine the stoichiometry of binding, 100 µM of [3-^{3}H]26,27-DHZ was incubated with 0.83 µM SMT in Buffer A for 45 min at 30 ^{0}C. Assay of an aliquot of the incubation mixture pre-equilibrated with inhibitor confirmed the loss of 99% of the orginal activity. The incubation mixture was dialyzed for 48 hr. against Buffer A, and then subjected to repeated ultrafiltration using an Amicon concentrator exclusion membrane to give a final ratio of tritium activity to protein calculated to correspond to 0.50 ± or 0.10 equiv of inhibitor per mole of enzyme (native enzyme as the tetramer). The above results are readily explained by a mechanism-based inactivation

process as shown in path *d*, Scheme 2. Experiments to establish the mechanism of inactivation and to determine the site of covalent protein modification are in progress.

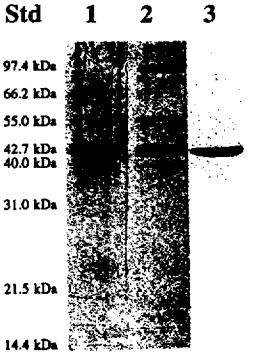


Figure 3. Affinity labeling of yeast SMT expressed in *E. coli*. Lane 1, SDS-PAGE (7.5%) gel of native protein stained with Coomassie blue (CB): Lane 2, [3-^{3}H]26,27-DHZ-SMT adduct stained with CB. Lane 3, fluorogram of [3-^{3}H]26,27-DHZ-SMT adduct.

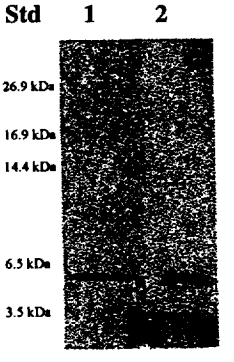


Figure 4. SDS-PAGE gel (20%) of [3-^{3}H]26,27-DHZ peptide adduct from tryptic digest. Lane 1, [3- ^{3}H]-26,27 -DHZ - peptide adduct stained with Coomassie blue. Lane 2, fluorogram of [3-^{3}H]26,27-DHZ-peptide adduct.

References and Notes

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Acknowledgment: The support of the Welch Foundtion (# D-1276) is appreciated.