Active Site Mapping and Substrate Channeling in the Sterol Methyl Transferase Pathway

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Running Title: Affinity-labeled sterol methyl transferase
SUMMARY

Sterol methyl transferase (SMT) from *Saccharomyces cerevisiae* was purified from *Escherichia coli* BL21(DE3) and labeled with the mechanism-based irreversible inhibitor [3-^3^H]26,27-dehydrozymosterol (26,27-DHZ). A 5-kDa tryptic digest peptide fragment containing six acidic residues at positions Glu64, Asp65, Glu68, Asp79, Glu82, and Glu98 was determined to contain the substrate analog covalently attached to Glu68 by Edman sequencing and radioanalysis using C18 reverse phase HPLC. Site-directed mutagenesis of the six acidic residues to leucine followed by activity assay of the purified mutants confirmed Glu68 as the only residue to participate in affinity labeling. Equilibration studies indicated zymosterol and 26,27-DHZ were bound to native and Glu68Leu mutant with similar affinity, whereas AdoMet was bound only to the native SMT, $K_d$ of ca. 2 µM. Analysis of the incubation products of the wild-type and six leucine mutants by GC-MS demonstrated zymosterol was converted to fecosterol, 26,27-DHZ was converted to 26-homo-cholesta-8(9),23(24)E,26(26')-trienol as well as 26-homocholesta-8(9),26(26')-3β,24β-dienol, and in the case of Asp79Leu and Glu82Leu mutants, zymosterol was also converted to a new product, 24-methylzymosta-8,25(27)-dienol. The structures of the methylenecyclopropane ring-opened olefins were determined unambiguously by a combination of $^1$H- and $^{13}$C-NMR techniques. A $K_m$ of 15 µM, $K_{cat}$ of 8 x 10^{-4} s^{-1} and partition ratio of 0.03 was established for 26,27-DHZ, suggesting the methylenecyclopropane can serve as a lead structure for a new class of antifungal agents. Taken, together, partitioning that leads to loss of enzyme function is the result of 26,27-DHZ catalysis forming a highly reactive cationic intermediate that interacts with the enzyme in a region normally not occupied by the zymosterol high-energy intermediate as a consequence of less than perfect control. Alternatively, the gain in enzyme function resulting from the production of a ∆25(27)-olefin originates with the leucine substitution directing substrate channeling along different reaction channels in a similar region at the active site.
INTRODUCTION

The sterol methyl transferases (SMTs) are appealing targets in the design of enzyme inhibitors, since they lie on a pathway with no counterpart in human physiology (1, 2). These enzymes are unique for catalyzing the biosynthesis of 24-alkyl sterol membrane inserts in microorganisms (3, 4). Several substrate analogs have been designed to mimic the topological and electrostatic features of the transition state for the C-methylation reaction and several of these compounds, including 24(R,S),25-epiminolanosterol and 25-aminolanosterol (5), markedly inhibit growth of microbes associated with human disease (6-9).

The SMT from the yeast Saccharomyces cerevisiae has been cloned and overexpressed in Escherichia coli BL21(DE3) cells, and purified to homogeneity (10, 11). The enzyme is a tetramer of molecular weight 172,000 Da and its activity is subject to down-regulation by ergosterol and up-regulation by ATP (1). Comparison of protein sequences deduced from the cDNA for fungal SMTs (Fig.1) revealed a highly conserved region rich in aromatic amino acids, referred to as Region I, containing a signature motif Y81EYGWG86 not present in other AdoMet-dependent methyl transferases. As each of these enzymes uses zymosterol 1A as the sterol acceptor molecule, the aromatic-rich domain of Region I has been proposed to be involved in substrate binding, and possibly product formation, by stabilizing intermediate carbenium ions generated during sterol C-methylation (11). A general stereochemical model (the “steric-electric plug” model) for the coupled Si-face (β-or back face attack) C-methylation of the zymosterol Δ24-bond 1A and subsequent deprotonation of C-28 with concerted migration of hydrogen to fecosterol 3A has been proposed based primarily on differential inhibition and isotopic labeling studies as well as recent investigations involving product outcomes resulting from site-directed mutagenesis experiments on SMT action (12-14).
A central feature of the steric-electric plug model is the predicted intermediacy of a high energy intermediate 2 which can be converted to a $\Delta^{24(28)}$- or $\Delta^{25(27)}$-olefinic product (Scheme 1). The usual paradigm for the formation of sterol side chain olefins such as fecosterol from zymosterol in fungi or cyclolaudenol 4B and cyclosadol 5B from cycloartenol 1B in algae and vascular plants, respectively, requires distinct SMTs to generate the variant isomers. However, recent studies with SMTs from fungi and plants employing a range of sterol acceptor molecules and isotopically branching sensitive experiments evoke a common set of enzymatic intermediates involved with the stereochemically related events-electrophilic alkylations, rearrangements and deprotonations (14, 15). Since no gene encoding a protein $\Delta^{23(24)}$- or $\Delta^{25(27)}$-SMT activity has been previously identified we were unable to identify consensus motifs which might contribute to the unique regioselectivity of the catalysts. Instead, we performed site-directed mutagenesis of a highly conserved aromatic amino acid residue in Region I, Tyrosine-81, to establish whether Region I contributes to regiocontrol in the reaction. Unexpectedly, the Tyr81Phe mutant exhibited altered substrate specificity catalyzing the successive C-methylation of desmosterol 1C to 24(28)-methylene cholesterol 3C and to a mixture of multiple doubly-alkylated sterols found in primitive vascular plants (16), including isofucosterol 7C, fucosterol 8C and clerosterol 9C. The Tyr81Phe mutant SMT failed to generate a mono-alkyl structure other than the $\Delta^{24(28)}$-methylene product from a $\Delta^{24(25)}$-substrate.

A major determinant which controls product diversity is believed to be the precise conformation of the sterol side chain at the sterol binding site (14, 17). Although many of the mechanistic and stereochemical details of the C-24 alkylation pathways have been verified (18, 19), little is known about the active site of any SMT or the manner in which a SMT enzyme...
imposes a particular conformation on its acceptor molecule, precisely controls the coupled-methylation deprotonation reaction and establishes kinetic and substrate specificity for C₁- and the successive C₁-methyl transfers ultimately giving rise to the side chains of fungal ergosterol and plant sitosterol, respectively. We recently reported the first potent mechanism-based inactivator of *S. cerevisiae* SMT, 26,27-dehydrozymosterol (26,27-DHZ), which made it possible to stoichiometrically and covalently modify the active site of the enzyme (20). A tryptic digest fragment containing the inhibitor-peptide adduct was sequenced and found to be contiguous with Region I. The mechanism of inhibition and covalent attachment of 26,27-DHZ responsible for trapping an active-site nucleophile suggested nucleophilic attack of the ∆²₄-bond of the S-methyl group of AdoMet leading to a reactive ring opened intermediate-∆²₅(2₇)-olefin 12, shown in Scheme 2, in analogous manner to the mechanism proposed for the catalysis of 6-cyclopropylidene-3E-methyl-hex-2-en-1-yl-diphosphate for monoterpene cyclase (21).

In this paper, we demonstrate that 26,27-DHZ reacts specifically with Glu68. The structure of the activated 26,27-DHZ intermediate that leads to enzyme inactivation and the structure of the C-methylation turnover product reveal a novel mode of C-methylation catalysis that is different from the mechanism of irreversible inactivation of monoterpene cyclase by 6-cyclopropylidene-3E-methyl-hex-2-en-1-yl-diphosphate. In addition, we describe product analyses and kinetic results for site-directed mutagenesis studies that implicate key acidic amino acids in Region I, themselves not required for catalytic activity, capable of directing substrate channeling to produce multiple olefins.

*<Insert Scheme 2 here>*
EXPERIMENTAL PROCEDURES

Materials—The sources of reagents and substrates: zymosterol, fecosterol, 26,27-dehydrozymosterol, [3-^3H]26,27-dehydrozymosterol ([3-^3H]26,27DHZ, 9.0 µCi/µmol), [^3H]-methyl]AdoMet (10.8 µCi/µM),[^3H-methyl]AdoMet (99.3 % enrichment), and MSD isotopes, and chromatographic materials were as described in our preceding papers (12, 21). An antibody to the pure S. cerevisiae SMT was a gift of Dr. M. Venkatramesh at Monsanto Co and purified according to the manufacturer’s protocol using a serum IgG purification kit supplied by Bio-Rad.

General Methods—General methods for spectroscopic analysis, heterologous expression and homogenate preparation and analysis, steady-state kinetic experiments and protein purification were as described (12, 13). The initial velocity data were determined using a Sigma plot 2001 plus Sigma enzyme kinetic program (SSPS Inc). Measurement of $K_{m(app)}$ and $V_{max(app)}$ for sterol employed a concentration range of 5 µM to 200 µM. Data were fitted to the equation, $v = V_{max}^* (S/K_m + S)$, using a nonlinear least square approach. Kinetic constants possessed ± standard errors of ± 5 % and $R^2$ values = 0.95 to 0.97. GC-MS analyses were performed on a Hewlett-Packard 6890 gas-chromatograph-mass spectrometer. Routine protein concentrations were estimated according to the method of Bradford with commercial reagents (Bio-Rad) using bovine γ-globulin as standard (23).

Plasmid construction of mutant SMTs—Point mutations were generated using the QuikChange™ site-directed mutagenesis kit (Stratagene) according to the manufacturer’s instructions, which includes the use of the proofreading thermostable DNA polymerase from Pyrococcus furiosus. Mutagenic oligonucleotides had 23-28bp overlap with the cDNA sequence before and after the mutated codon, and a melting temperature equal or greater than 78°C (as per Stratagene’s instruction manual). Oligonucleotide sequence of mutagenic primers is listed in
Table 1. The recombinant pET23a-ERG6 was used as the mutagenesis template. Mutations were introduced into bp 161-163, 163-165, 271-273, 306-308, 315-317, and 363-365 of ERG6 wild type cDNA, to generate six mutant ERG6 proteins. The mutant ERG6 proteins had the following single amino acid changes: Glu64Leu, Asp65Leu, Glu68Leu, Asp79Leu, Glu82Leu, and Asp98Leu. Following DpnI-treatment of the mutagenic PCR product, the mutant plasmids were transformed into BL21(DE3) competent cells (from Novagen). All the mutations were verified by automated DNA sequencing to ensure that only the desired mutations and no other changes were inserted into the cDNA.

<Insert Table 1>

Expression and assay of native and mutant SMTs—Native and engineered proteins were expressed in BL21(DE3) cells as previously described (14) with minor modification as follows. Briefly, transformed cells in BL21(DE3) cultures were grown in LB medium containing 50 mg/ml of ampicillin overnight. Harvested cells were lysed using a French press, followed by generation of a 100,000 x g supernatant used for analysis of product distribution. The 100,000 x g fraction was purified to apparent homogeneity for $K_{cat}$ determinations as described (14). The initial velocity versus substrate concentration curves for the SMT catalyzed C1-transfer reaction were determined using a fixed $[^3H_2\text{-methyl}]$AdoMet concentration of 50 µM and varying the concentration of sterol at 5, 10, 15, 25, 75, 100, 150, and 200 µM sterol (final concentration). The two substrates dissolved in Tween 80 (1.0%, v/v) were incubated with 0.68 µM total protein at 35°C for 45 min. The conversion of zymosterol to products was assayed by counting total radioactivity in the non-saponifiable lipid fraction (NSF) of the quenched enzyme assay and plotted according to the method of Lineweaver and Burk. Further assessment of the enzyme generated products was by GC-MS analysis of the NSF fraction of activity assays performed overnight at saturating levels of zymosterol and AdoMet.
**Detection of SMT proteins**—Aliquots of total and soluble (100,000 x g supernatant) protein from crude *E. coli* homogenates harboring native or mutant cDNA of the SMT, prepared as described (14), were separated on an 12% SDS-polyacrylamide gel and stained for total protein using Coomassie blue. The stained gels were scanned with a LKB densitometer, and the density of the IPTG inducible band (corresponding to the predicted molecular weight for the SMT protein of ca. 43 kDa) was determined as a percentage of the total density of the scan. In addition, Western Blot analysis for the expressed proteins using the native SMT antibody was performed under the same chromatographic conditions to estimate SMT levels (14).

**The inhibitor-peptide adduct**—Chemical affinity labeling using pure SMT (0.83 µg), AdoMet (100 µM) and [3-^3H]26,27DHZ (100 µM) was as described (19). The affinity-labeled protein was digested with trypsin and the resulting inhibitor-peptide adduct separated using 20% SDS-PAGE gel system. The amino acid sequencing of the isolated radioactive 5-kDa fragment (2.5 x 10^5 dpm) was carried out on an aliquot (2.5 x 10^4 dpm) of the excised material using a Porton (Beckman) automated sequencer with on-line Beckman Gold HPLC. Standard Beckman gradient protocols for the microPTH column system were followed for phenylthiohydantoin-amino acids separation and identification. Radioactivity of sequencing eluates was determined by liquid scintillation counting (Beckman, LS-6500).

**Binding assay**—Determination of the dissociation constant (*K_d*) for zymosterol and AdoMet was performed by the filtration method, adapted from reference 24. Briefly, *K_d* for the two ligands was established using pure native and mutant SMT expressed in BL21(DE3) cells. Assay conditions for AdoMet involved dissolving the ligand in a 1.7 ml reaction vessel containing 100 µl buffer A (50 mM TrisHCl (pH 7.5), 2 mM MgCl₂, 1 mM EDTA, 2 mM β-mercaptoethanol and 5% glycerol) at pH 7.5, 0.57 µM SMT and incubated at 32°C. For sterol, Tween 80 (1% w/v) was added to the vial to assist solubilizing the sterol. The *K_d* for AdoMet and sterol was
determined by aliquoting 0.04 µM to 3.76 µM and 0.25 µM to 10 µM, respectively, to the reaction vessel. After 2.5 h of equilibration on a level shaker, the protein-ligand complex was put on a GF/B glass filter disk (disks were 2.5 cm diameter, Whatman). The filter disks were pretreated with 1 mg/ml BSA solution at 4°C overnight to prevent nonspecific binding of the ligand to the disk. Ligand bound proteins were washed 3 times, 500 µM each, with ice-cold buffer A. The disk was air dried and counted by liquid scintillation to determine the amount of radioactivity that remained on the disk from which the amount of bound ligand was determined. Non-specific binding was determined in the presence of 100 fold of excess unlabeled AdoMet or sterol. At the specified concentration of ligand, all potential sites of the enzyme are assumed to be saturated and any radioactivity recovered from the assay was viewed to be from non-specific binding. In no case, was non-specific binding greater than 10%. Binding of AdoMet was independently performed in two 5-cell Scienceware, H420260-0000, equilibrium dialyzer at 32°C. Equilibration conditions employed 1 ml buffer A, in each chamber, and 0.57 µM of purified enzyme. The $K_d$ value for AdoMet in the presence of SMT was determined by transferring 0.07 µM to 3 µM $[^3H_{-methyl}]$-AdoMet to the buffer chamber and SMT to the protein chamber. Each side of the cell was separated by Spectra/Por membranes with cut-off $M_r = 6,000$ to 8,000. After a 8-h incubation on a level-shaker, aliquots were recovered from each chamber, and the radioactivity measured in Beckman LS6000 liquid scintillation analyzer to determine the amount of radioactivity in the buffer chamber ([AdoMet]$_{\text{free}}$) and the protein chamber ([AdoMet]$_{\text{free}}$ + [AdoMet · SMT]). The calculated [AdoMet]$_{\text{free}}$ was subsequently subtracted from the calculated concentration of bound SMT. The data were presented in hyperbolic form (equation 1). The bound ligand (AdoMet or sterol) concentration $B$ was plotted as a function of free ligand concentration $[L]_f$. The $K_d$ value was estimated using a computer program (Sigma plot 2001 plus Ligand binding Marco SSPS, Inc) by non-linear least-squares analysis of $B$ versus $[L]_f$ plot
fitting to the standard Langmuir type binding equation; $B = \frac{B_{max}}{1 + \frac{K_d}{[L]_f}}$ (equation 1), where $B$ is [ligand · SMT] and $[L]_f$ is [ligand]free. The equation assumes that there is only one ligand-binding site on the enzyme.
RESULTS

Identification of the amino acid residue labeled with [3-^3H]26,27-DHZ—In order to localize the site affinity labeled with the mechanism-based inactivator in the primary structure, a trypsin digest fragment of ca. 5-kDa was prepared from SMT that had been treated with [3-^3H]26,27-DHZ. The labeled 5-kDa peptide was subjected to Edman degradation to give an unambiguous sequence of 19 amino acids. The radioactivity associated with the covalently attached irreversible inhibitor was detected between the fifth to tenth cycles (ca. 80% of the tritium injected into the column was recovered in these fractions) with the major peak of radioactivity eluting in the seventh sequencing cycle (Fig. 2). On the basis of these results, we concluded that Glu68 was specifically labeled with the inhibitor and represents the putative acidic amino acid residue attached to [3-^3H]26,27-DHZ. However, since the 5 kDa trypsin digest fragment is expected to contain 45 amino acids and six acidic residues at positions Glu64, Asp65, Glu68, Asp79, Glu82, and Asp98 (Fig. 2), it was unclear whether the two additional acidic amino acid residues at positions Glu82 and Glu98 not involved in the Edman sequencing of the peptide might also be affinity labeled with the irreversible inhibitor. Therefore, additional experiments were carried out to confirm Glu68 as the only residue attached to [3-^3H]26,27-DHZ in the SMT.

Effects of mutations of acidic residues on catalysis and binding affinity—In the initial series of experiments it was of interest to establish whether any of the acidic amino acids in the tryptic digest were catalytically dysfunctional. A set of SMT mutants were constructed by site-directed mutagenesis in which the relevant acidic amino residue in the tryptic digest fragment was replaced with leucine. Each of the resulting mutants was expressed in BL21(DE3) cells to similar levels as the wild-type cDNA as revealed by Western Blot. The mutant enzymes were subsequently purified in a similar manner to homogeneity, suggesting that none of the mutant
proteins were significantly altered conformationally. The steady-state kinetic parameters determined using zymosterol as the sterol acceptor molecule for the mutants are reported in Table 2. Although there are differences in the catalytic competence amongst Glu86Leu, Asp65Leu, Glu79Leu, Glu82Leu and Glu98Leu mutants, they were active whereas Glu68Leu was inactive, thereby indicating only Glu68 is a critical amino acid in the catalysis of 26,27-DHZ. When the amount of enzyme was increased by 10-fold, the Glu68Leu mutant continued to fail to exhibit appreciable activity. The rate of catalysis observed for the Glu98Leu mutant approached the lower limit of detection in our activity assay system. Nonetheless, we could measure the kinetic constants for 26,27-DHZ using the wild-type SMT which were $K_m$ of 15 µM and $K_{cat}$ of $8 \times 10^{-4}$ s$^{-1}$. From our earlier incubations with the recombinant yeast SMT, the $k_i$ and $K_{inact}$ of 1.1 µM and 1.52 min$^{-1}$, respectively were determined (20). The partition ratio, a measure of the production of product per inactivation event, can now be calculated from the ratio $k_{cat26,27-DHZ}/K_{inact26,27DHZ}$ to be 0.03, suggesting 26,27-DHZ to be a potent inhibitor of SMT action.

Binding experiments were performed to definitively confirm that the Glu68Leu mutant can bind sterol thereby confirming the structure of the mutant was not changed significantly by the amino acid replacement. As shown in Figure 3, zymosterol binds to the native SMT with a $K_d$ of ca. 2 µM and 26,27-DHZ binds with a similar $K_d$ value (data not shown). Scatchard analysis of the binding data revealed a single binding site for sterol (Fig. 3). AdoMet also binds to the native SMT with similar affinity as the sterols to SMT, $K_d$ of 0.5 ± 0.08 µM (Fig. 3). Equilibrium dialysis gave a similar binding isotherm for AdoMet generating a $K_d$ of each 4 µM. Based on the two methods, the $K_d$ of AdoMet is ca. 2 µM. By comparison, the $K_d$ for zymosterol and 26,27-DHZ to the Glu68Leu mutant was ca. 5 µM whereas AdoMet failed to bind at all (no saturation
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of the enzyme was evident). The leucine substitution appears to generate an impaired active center that prevents productive binding of AdoMet and this may be the reason for inactivity. Since the Tyr81Phe mutant can C-methylate fecosterol producing a mixture of 24-ethyl (and ethylidene) sterols (14), fecosterol was tested as a substrate with each of the leu mutants. In no case was fecosterol a suitable substrate for SMT action.

<Insert Figure 3 here>

Identification of Products of Zymosterol Catalysis—The product profiles of the six mutants assayed with zymosterol are summarized in Table 2. Each of the active mutants produced fecosterol 3A and in the case of Asp79Leu and Glu82Leu was accompanied by a second olefin which in GLC eluted earlier than fecosterol (retention time relative to cholesterol of 1.16 3A; molecular weight \( m/z = 398 \)) as shown in Figure 4. The mass spectrum of the new sterol is identical to an authentic specimen of 24-methyl zymosta-8,25(27)-dienol (retention time relative to cholesterol of 1.14; molecular weight \( m/z = 398 \) 4A) generated in a cell-free preparation from the algal Prototheca wickerhamii (25). Further evidence for the biosynthesis of the new sterol was by incubation of Asp79Leu mutant with saturating amounts of \([\text{2H}_3\text{-methyl}]\)AdoMet and zymosterol and analysis of the activity assay products by GC-MS. Both fecosterol and 24-methyl zymosta-8,25(27)-dienol were labeled with deuterium from \([\text{2H}_3\text{-methyl}]\)AdoMet. \(^2\text{H}\)-Labeled fecosterol possessed a two mass unit increase compared to the native fecosterol with a molecular weight of \( m/z = 400 \) (M\(^+\) 100\% and other diagnostic ions at M\(^+\) - CH\(_3\), 385 and M\(^+\) - CH\(_3\) - H\(_2\)O, 367). By contrast, \(^3\text{H}\)-labeled 24-methyl zymosta-8,25(27)-dienol possessed a three mass unit increase compared to the native 24-methyl zymosta-8,25(27)-dienol with a molecular weight of \( m/z = 401 \) (M\(^+\) 100\% and other diagnostic ions at M\(^+\) - CH\(_3\), 386 and M\(^+\) - CH\(_3\) - H\(_2\)O, 368). The results of the deuterium incorporation studies into the sterol side chain are consistent with the
Affinity-labeled sterol methyl transferase formation of a 24(28)methylene structure (2\(^2\text{H}\)-atoms at C-28) and a \(\text{CH}_3\) structure (3 \(^2\text{H}\)-atoms at C-28) thereby generating olefins \(3\text{A}\) and \(4\text{A}\), respectively.

Identification of Products of 26,27-DHZ Catalysis—In our earlier study of 26,27-DHZ assayed with yeast SMT (12, 20), we failed to detect any product formation by GC-MS or HPLC. At the time, we were operating on the assumption that 26,27-DHZ would elute earlier in chromatography than its putative turnover product-12 in a manner analogous to the GLC behavior of zymosterol to fecosterol (Fig. 4). However, upon further study, we detected two unknown compounds in a 3 to 1 ratio that are generated in 2% yield from the saponification mixture of SMT assayed with 26,27-DHZ. From a preparative scale incubation with AdoMet and 26,27-DHZ, two unknown compounds were detected in the HPLC chromatogram (Phenomenex) eluting before (\(\alpha_c\) 0.35, RRTc 1.72, \(M^+\) 414) and coincidental (\(\alpha_c\) 0.68, RRTc 1.29, \(M^+\) 396) with the substrate 26,27-DHZ (\(\alpha_c\) 0.68, RRTc 1.29; \(M^+\) 382). The compounds eluting at \(\alpha_c\) 0.35 (dil) and 0.68 (monol) were subsequently purified to homogeneity using a second HPLC column (TohoHaas). NMR analyses of the enzyme-generated products indicated retention of the zymosterol nucleus for both the monol and diol structures (data not shown). The relevant chemical shifts of the side chain proton atoms in the 500 MHz spectrum of the monol showed the presence of two exomethylene protons (\(\delta\) 4.70 (s, 1H), 4.71 (s, 1H) and two additional olefinic protons \textit{transoid} to one another resonating at \(\delta\) 5.41 (1H, m) and 5.40 (dd, 12.8, 5.7 Hz) and an allylic methyl resonating at \(\delta\) 1.71 (3H, s). The coupling constants for reference to \textit{transoid} and \textit{cisoid} olefinic protons are reported reference 26. The coupling network determined from the COSY and TOCSY and the \textsuperscript{13C} NMR indicated two double bonds in the side chain between C-23 and C-24 and C-26 and C-27. Side chain carbon atoms for the monol: \(^1\text{H}\) NMR
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(500 MHz, CDCl$_3$) $\delta$ (ppm) 1.48 (m, C-20), 0.93 (3H, d, 6.4 Hz, C-21), 1.76 (m, C-22) and 2.14 (m, C-22), 5.41 (m, C-23), 5.40 (dd, 12.8, 5.7 Hz, C-24), 2.69 (2H, d, 5.7 Hz, C-25), 4.70 (s, C-27), and 4.71 (s, C-27), 1.71 (3H, s, C-28); $^{13}$C NMR (125 MHz, CDCl$_3$) $\delta$ ppm 36.6 (C-20), 18.7(C-21), 39.1 (C-22), 129 (C-23), 130.4 (C-24), 41.3 (C-25), 145.4 (C-26), 110.2 (C-27), 22.4 (C-28). The UV spectrum of the monol (in EtOH) showed end absorption, confirming that the double bonds in the side chain were not in conjugation. Incubation of 26,27-DHZ paired with $[^2H_3$-methyl]AdoMet in the presence of SMT generated a deuterated monol with a molecular weight of M$^+$ 399, suggesting methyl to methylene transformation failed to occur during the C-methyl transfer reaction. The tri-deuterated methyl group was assigned to the terminal allylic methyl group at C-26 by the disappearance of the signal resonating at $\delta$ 1.71 in the $^1$H NMR spectrum which is assigned to C-28 in the product. Thus C-28 is derived from the methyl group attached to AdoMet. The monol is assigned structure 18.

The $^1$H NMR spectrum of the side chain of the unlabeled diol showed the presence of two exomethylene protons ($\delta$ 4.81 (1H, s), 4.90 (1H, s)), a proton geminal to the alcohol group ($\delta$ 3.66 (1H, m) and an allylic methyl group ($\delta$ 1.77 (3H, s)). Side chain carbon atoms for diol: $^1$H NMR $\delta$ (ppm) 1.44 (m, C-20), 0.96 (3H, d, 6.6 Hz, C21), 1.05 (m, C-22), and 1.57 (m, C-22), 1.38 (m, C-23) and 1.52 (m, C-23), 3.66 (m, C-24), 2.24 (dd, 2.9 and 13.6 Hz, C-25), and 2.07 (dd, 9.4 13. 6 Hz, C-25), 4.90 (s, C-27), and 4.81 (s, C-27),1.77 (3H, s, C-28); $^{13}$C NMR $\delta$ (ppm) 36.3 (C-20), 18.8 (C-21), 31.8 (C-22), 33.6 (C-23), 69.2 (C-24), 46.1 (C-25), 142.9 (C-26), 113.5 (C-27), 22.4 (C-28). The COSY and TOCSY correlation indicated connectivity between the proton at $\delta$ 3.66 ppm and a pair of protons at C-23 ($\delta$ 1.38, 1.52) and C-25 ($\delta$ 2.07 and 2.24), suggesting the hydroxyl group in the side chain was located at C-24. In addition, the protons associated with C-25 shows connectivity with the exomethylene at C-27 and the allylic methyl
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group at C-26, suggesting the “extra methyl group” was attached to C-26 rather to C-24. The mass spectrum of the deuterated diol following incubation with [\(^{2H3}\)-methyl]AdoMet contained diagnostic fragments: M\(^+\) 417; M\(^+\)-CH\(_3\), 402; M\(^+\)-H\(_2\)O, 399; M\(^+\) - 33, 384), consistent with the diol assigned to structure 20 (Scheme 3).

There was insufficient material to establish the stereochemistry of the hydroxyl group at C-24 unambiguously. Therefore, the diol was prepared synthetically from the C-24 aldehyde of zymosterol C-3 acetate as outlined in Scheme 4. Aldehyde 21 was prepared as described in ref. 27 and the general coupling method to generate 22 was as described in ref. 28. The resulting diastereoisomeric mixture of 20 and 23 was resolved by HPLC (Tosohaas TSK gel, ODS-120A, 90% MeOH/H\(_2\)O). The two compounds were distinguished from each other by their relevant physical, chromatographic and spectral characterizations: 24α-hydroxy 23 (HPLC, 36.0 min; mp, 136-137\(^{\circ}\); \([\alpha]\)\(_D\), +83.7; \(^1\)H NMR C-21, \(\delta\) 0.94, d, \(J = 5.5\) Hz) and 24β-hydroxy 20 (HPLC, 38.5 min; mp, 127-128\(^{\circ}\); \([\alpha]\)\(_D\), +32.0; \(^1\)H NMR C-21, \(\delta\) 0.96, d, \(J = 6.6\) Hz).

The Mosher (\(^1\)H) method was employed to resolve whether the enzyme generated diol contained the 24α- or 24β-hydroxy group (29). The complete proton assignment for both the 2-methoxy-2-phenyl-2(trifluoromethyl)acetic acid (MTPA) (S)- and (R)-esters of synthetically prepared 20 were determined by extensive 2D NMR analyses (data not shown). The \(\delta\) (\(\delta\)\(_S\) - \(\delta\)\(_R\)) values shown in Scheme 5 for the (S)- and (R)-MTPA indicate that the configuration of the 3-hydroxy and 24-hydroxy groups are β-oriented. Since the enzyme-generated diol co-elutes with
the synthetic diol used to prepare the MTPA esters, the configuration of the 24-hydroxyl group in the natural product must be β-oriented. The Asp79Leu and Glu82Leu mutants were assayed with saturating amounts of 26,27 DHZ and AdoMet overnight. The resulting product yield and ratio of monol and diol were equivalent to a similar activity assay performed with wild-type enzyme.
DISCUSSION

According to the steric-electric plug model, C-methylation of zymosterol by yeast SMT is to occur via a non-covalent pathway whereby methyl addition to $\Delta^{24}$ and deprotonation of C-28 gives rise to a nucleophilic rearrangement in which H-24 migrates to C-25 on the Re-face of the substrate double bond in concert with the initial ionization. Kinetic studies indicate a random Bi Bi mechanism for sterol C-methylation (22). Until recently, few details of protein structure were available that would lend credence to the hypothesized mechanism. It was proposed that a set of aromatic amino acids in the active site would direct folding of the sterol side chain into an appropriate catalytic conformation for C-methylation and stabilize the positively charged high energy intermediate during rearrangement (12, 17). In addition, it was proposed that a carboxylate anion would serve as the deprotonating agent of C-28 methyl and concurrently act as an enzymatic counterion to AdoMet (30).

To date, however, no SMT has been crystallized therefore no three-dimensional structure is available to assist in the analyses. We therefore conducted further affinity labeling using 26,27-DHZ and site-directed mutagenesis experiments with the yeast SMT to determine the precise location of a general base in the active site and to gain information on the role of acidic amino acids in the tryptic digest fragment. Our observation that 26,27-DHZ binds irreversibly to the Glu68 of yeast SMT suggests the residue is situated in a subsite of the active center. The formation of fecosterol by the leucine mutants Glu64, Asp65, Asp79, Glu82, and Glu98 but not by the Glu68Leu mutant, offers additional support for the involvement of Glu68 in catalysis of sterol acceptor molecules, including 26,27-DHZ.

The C-methylation of 26,27-DHZ was directional, that is, it was initiated through a backside nucleophilic attack of a methyl group on the methylenecyclopropane leading to ring opening and ring expansion. The termination step gives rise to either olefinic (elimination) or hydroxyl (water
addition) products. Ring strain and the rich \( \pi \)-electron density associated with the overlapping \( \text{sp}^2 \)-systems of the monosubstituted cyclopropane ring of 26,27-DHZ make the cyclopropanoid susceptible to C-methylation from AdoMet and nucleophilic rupture leading to selectivity with respect to the direction of bond cleavage (Scheme 3). We propose the C-methylation-elimination reaction involving 26,27-DHZ proceeds by the C-methylation of C-26 of substrate 10 followed by formation of intermediate 16 which can undergo corner-to-corner nucleophilic rearrangement and cyclopropane ring fission to generate chain extension. The resulting intermediate 17 can be deprotonated to generate 18 (path a), or intermediate 17 can be trapped by an active-site nucleophile 19, probably by a nonconcerted ionization of 17 (path b), thereby rendering the protein inactive. Stereospecific formation of diol 20 with a 24\( \beta \)-hydroxyl group and the \textit{transoid} orientation of the 23,24-double bond in 18 during enzyme-catalyzed transformation of 10 provides particularly strong support for the proposition that orientation effects related to the conformation of the side chain in binding or transformation are critical to the effectiveness of substrate analogs as SMT inhibitors (5, 14, 17). It appears most likely that elongation of the side chain by methylation of C-26 arrested the ongoing intramolecular rearrangements at the terminating carbocation and the final deprotonation of C-23 yielded 26-homo-cholesta-8(9),23(24)\( E \),26(26')-trienol 18. The abortive C-methylation product, diol 20, thus generated by catalysis of 26,27-DHZ and released from the enzyme by saponification is diagnostic of the structure and stereochemistry of the normal enzyme-bound intermediates. A water bridge between the Glu68 and the cationic intermediate can also form. In which case, formation of diol 20 may result from the addition of water to the cationic intermediate 18. The novel methylenecyclopropane rearrangement catalyzed by SMT reported for the first time is unrelated to cyclopropyl sterol synthesis in marine organisms (31). The mechanism for ring opening of the
methylenecyclopropane of 26,27-DHZ is distinctly different from the mechanism involved with methylenecyclopropane inactivators studied in fatty acid metabolism (32).

The detection of the 24β-OH group in diol 20 suggests the alkylated enzyme species was formed from the Si-face of the original substrate double bond, on the side of the 24,25-double bond where the carboxylate anion should reside for mechanistic reasons. However, since Glu68 is likely involved with 23-deprotonation it must be spatially distal to the nucleophile involved with C-28 deprotonation. The cyclopropane ring of 26,27-DHZ by virtue of its ring strain and electron-donating substituent provides structural features absent from zymosterol that upon catalysis of the acceptor molecule will allow for Glu68 to be brought into range of this rotationally flexible side chain structure.

The exact nature of Glu68 involvement in zymosterol catalysis is not clear. The generation of 26-homo-cholesta-8(9),23(24)E,26(26′)-trienol 20 from catalysis of 26,27-DHZ by native SMT suggests Glu68 acts as a cryptic base in C-23 deprotonation. The failure of AdoMet to bind to the Glu68Leu mutant suggests the acidic amino acid may interact with AdoMet in the active center of the native enzyme as a counter ion. Of interest, is the $K_d$ of AdoMet for the SMT was 2 µM similar to the $K_d$ of 11 µM for the AdoMet: glutamyltransferase from Salmonella typhimurium (33).

It seems unlikely that Glu68 serves as the same active site base responsible for deprotonation in formation of fecosterol from zymosterol since no $\Delta^{23}$-sterols are formed by the yeast SMT. Given that Glu68 is a non-conserved residue in fungal SMT sequences and 26,27-dehydrocycloartenol 10B fails as a mechanism-based inactivator of the alga Prototheca wickerhamii SMT indicate the existence of subtle differences between the two groups of SMTs, perhaps due to sequence variations at or near Glu68. Since the methylenecyclopropane structure is a unique inhibitor for S. cerevisiae SMT, it holds the potential to serve as a lead for the
rational design of species-specific agents aimed at modulating SMT activity, with a view to control and/or regulate ergosterol homeostasis in the cell.

The high degree of sequence conservation for the signature motif of Region I in fungi supports a catalytic function for this domain. Region I is also found in plants with a similar consensus sequence(14). Site-directed mutagenesis experiments of the acidic residues of the tryptic digest fragment of the yeast SMT provided additional support that Region I has a functional role in catalysis and that these specific amino acids are not deprotonating agents involved with the conversion of zymosterol to fecosterol. Of the six acidic amino acids in the tryptic digest fragment, only the leucine mutants of Asp79 and Glu82 elicited substrate channeling. The Asp79 and Glu82 mutants furnished, in addition to fecosterol, a novel C-methyl sterol product with the $\Delta^{25(27)}$-olefin structure, The deprotonation leading to the formation of 24-methyl zymostan-25(27)-dienol ($\Delta^{25(27)}$-olefin) involves protons chemically and geometrically distinct from that lost in the generation of fecosterol ($\Delta^{24(28)}$-olefin) indicating a critical feature of enzymic control that can lead to multiple olefin production by a fungal SMT. The isosteric interchange of aspartate or glutamate with leucine make the active site region less compact. A somewhat loosely packed active center may be more flexible thereby allowing the sterol side chain to develop interactions with polar amino acids that otherwise would not be contacted during catalysis.

In summary, we observed that the yeast SMT active center, normally generating a single olefin, has the requisite amino acids to give rise to multiple olefins that occur naturally in plants. Introduction of the Asp79Leu and Glu82leu mutations into the SMT active site results in the formation of a mixture of $\Delta^{24(28)}$- and $\Delta^{25(27)}$-olefins as the final products, suggesting hydrophobic residues in the binding pocket can affect partitioning through a common high energy intermediate 2 in the SMT. The acidic amino acids Asp79 and Glu82 must lie on the $\alpha$-
face of the sterol side chain at the time of binding otherwise they would abstract the proton from H-24 during the 1,2-hydride shift involved with the coupled methylation-deprotonation reaction catalyzed by SMT. The Glu68 which interacts with the sterol side chain β-face appears to be essential for catalysis but it is not a likely acidic amino acid unit for C-28 deprotonation in zymosterol conversion to fecosterol. Finally, it should be noted that differences in the amino acid composition of Region I that exist in plants compared to fungi may contribute to the different sterol specificities and product mixtures catalyzed by SMT isoforms.

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FIGURE LEGENDS

Fig. 1. Comparison of 26,27-dehydrozymosterol binding site sequence of S. cerevisiae SMT with related sequences from other fungi. Note the alignment of the aromatic-rich signature motif YEYGWG (boxed), the non-conserved Glu68 affinity labeled with 26,27-DHZ and the six acidic amino acids (bold) mutated to leucine.

Fig. 2. (a) Amino acid sequencing and (b) radioassay for [3-³H]26,27-dehydrozymosterol-SMT peptide fragment after digestion.

Fig. 3. Langmuir isotherms for the determination of the dissociation constant ($K_d$) for zymosterol (panel A) or AdoMet (panel B) binding to the SMT via filter binding versus [ligand]$_{free}$ ($R^2 = 0.98$). Equilibration was performed as described under “Experimental Procedures”. The inset shows the Scatchard transformation of these data (6 out of 7 points are shown in the plot). Assays were performed with 0.25, 0.5, 1, 3, 5, 7, and 10 µM of zymosterol and 0.04, 0.07, 0.55, 0.73, 1.47, 1.84, 2.20, 2.57, 2.94, and 3.30 µM AdoMet; B: Bound ligand, F: Free ligand.

Fig. 4. Total ion chromatogram (15 m DB-5 capillary column) of the sterol mixture generated by wild-type (top/bold) and Asp79Leu mutant (bottom/dotted) assayed with zymosterol; peak 1: zymosterol, peak 2: 24-methyl zymosta-8,25(27)-dienol and peak 3: fecosterol.
| Genotype | Protein sequence and corresponding coding nucleotide sequence |
|----------|---------------------------------------------------------------|
| 1 E64L   | ACCGATAAAGATGCCGAAGAAGTCGCTCTCTGGATTATAATGAAGCCACACATTCCTACT |
| 2 D65L   | GATAAGAGATGCCGAAGAAGTCGCTCTGGATTATAATGAAGCCACACATTCCTACT |
| 3 E68L   | CGAAGAAGACGTCGCTCTTGAGGATTATAATTTTAGCCACACATTCCTACTTAACGTCGTTAC |
| 4 D79L   | ACACATTCCTACTATAACGTCGTTACACTGTTACTGATATGGTTGGGGTTCCTCTCTCTCTCTCTCT |
| 5 E82L   | ACTATAACGTCGTTACAGATTTCTATCTCTATATGGTTGGGGTTCCTCTCTCTCTCTCTCTCTCTCTCT |
| 6 E98L   | CTTTCCATTTCAAGAGATTTTATAAGGTGTAGGTGCTGCTGCCTGATAGCAAGACATG |
### TABLE II
Analysis of sterol methyl transferase mutants using zymosterol as a substrate.

| Strain     | $K_m$ (µM) | $K_{cat}$ (s$^{-1}$) | $K_{cat} / K_m$ (x 10$^{-4}$) | 3A | 4A |
|------------|------------|----------------------|-------------------------------|----|----|
| Wild Type  | 17         | 0.010                | 6.53                          | 100| 0  |
| Glu64Leu   | 13         | 0.005                | 3.59                          | 100| 0  |
| Asp65Leu   | 15         | 0.007                | 4.87                          | 100| 0  |
| Glu82Leu   | 22         | 0.005                | 2.10                          | 99 | 1  |
| Glu98Leu   | 74         | 0.001                | 0.12                          | 100| 0  |
| Glu68Leu   | NA$^1$     | NA                   | NA                            | NA | NA |
| Asp79Leu   | 17         | 0.010                | 5.48                          | 81 | 19 |
| Glu82Leu   | 22         | 0.005                | 2.10                          | 99 | 1  |

$^1$NA, no activity
|   | Species           | Sequence                      |
|---|------------------|-------------------------------|
| 1 | *S. cerevisiae*  | **RL**EDYN**E**AHSYNNVVTDF YEYGWG SSFHFSRFYK**GE**SFAASIAR |
| 2 | *C. albicans*    | RLNDYS**Q**LTHHYYNLVTDY YEYGWG SSFHFSRYYKGEAFRQATAR |
| 3 | *S. pombe*       | RIDGYK**S**VVNSYDLATDL YEYGWS QSFHFSRFYKGEAFQSIAR |
| 4 | *P. carinii*     | RFKFYA**T**LTRHYYNLVTDY YEYGWS TSHFCRFAKDESFSQAIAR |
| 5 | *N. crassa*      | RTKEYA**T**LTRHYYNLATDI YEYGWG QCFHFCRYSPIGESFYQAIAR |
| 6 | *A. fumigatus*   | RAVSNL**S**LL-SYYNLATDL YEYGWG TSFHFCRFAQEPFYQAIAR |
Scheme 1

A = zymosterol nucleus  
B = cycloartenol nucleus  
C = cholesterol nucleus  

Side chain

Path a
Path b
Path c
Scheme 3

C−28 Methyl from AdoMet

Path a

Path b

Alkylated enzyme species

KOH/MeOH

35
Scheme 4  Reagents and conditions: (a) 3-Bromo-2-methylpropene, Mg THF, RT, mixtures of 24-α/β hydroxyl (1:1), 80%; (b) HPLC purification, Nu: Zymosterol nucleus.
Scheme 5 Models of the MTPA esters of 20
Active site mapping and substrate channeling in the sterol methyl transferase pathway
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