Sterol Specificity of the *Saccharomyces cerevisiae ERG6* Gene Product Expressed in *Escherichia coli*

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ABSTRACT: The *ERG6* gene from *Saccharomyces cerevisiae* has been functionally expressed in *Escherichia coli*, for the first time, yielding a protein that catalyzes the bisubstrate transfer reaction whereby the reactive methyl group from (*S*)-adenosyl-L-methionine is transferred stereoselectively to C-24 of the sterol side chain. The structural requirements of sterol in binding and catalysis were similar to the native protein from *S. cerevisiae*. Inhibition of biomethylation was observed with fecosterol and ergosterol which suggests that ergosterol may function in wild-type yeast as a feedback regulator of sterol biosynthesis.
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The (*S*)-adenosyl-L-methionine:$\Delta^{24(25)}$-sterol methyl transferase (SMT: EC 2.1.1.41) synthesized by *Saccharomyces cerevisiae* catalyzes the stereoselective transfer of the (*S*)-adenosyl-L-methionine (AdoMet) methyl group to the 24,25-double bond of the sterol side chain (Fig. 1). The crucial role of this enzyme in regulating production of ergosterol, which functions as the fungal membrane insert, has stimulated considerable interest in the kinetic properties, stereochemistry, and mechanism of the transfer reaction (1–4), and in the design of inhibitors of SMT activity which may be used clinically or agrochemically as antifungal agents (5,6). The putative gene encoding the SMT from *S. cerevisiae* has been cloned and sequenced (7–9); however, there is no enzymological evidence to demonstrate that *ERG6* encodes an SMT enzyme. The purpose of this communication is to report for the first time enzymological evidence that *ERG6* is the yeast SMT structural gene and the sterol substrate specificity of the gene product.

MATERIALS AND METHODS

Media, strains, and plasmids. Escherichia coli AG-1 cells were used in transformation experiments. Plasmid pBR322 (10) was used as the cloning vector. Plasmid pRG458 (7) was provided by Dr. Martin Bard (Dept. of Biology, Indiana University, Indianapolis, IN) and used as the source of *ERG6* DNA. Cells were routinely maintained on Luria broth medium (11). Lucia broth medium was supplemented with ampicillin at a final concentration of 50 μg/mL when culturing cells that contained plasmid DNA. Cells were made competent using a solution of 50 mM $CaCl_2$ (11). SOB medium (12) was used for cell recovery following transformation with plasmid DNA.

Subcloning and DNA sequence analysis. DNA fragments generated by digestion of pRG458 with *Dra* I and *Nhe* I to isolate the open reading frame (ORF) were separated by agarose gel electrophoresis. The 1281 base pair fragment was eluted from the gel, extracted twice with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1), precipitated in ethanol, dried, and dissolved in *tris*-EDTA buffer (11). The DNA was made blunt-ended by incubation with T4 DNA polymerase and deoxynucleoside triphosphates and ligated into *Eco*R V-linearized, dephosphorylated pBR322. Protocols for plasmid and DNA fragment isolation, restriction endonuclease digestion, ligation, and transformation were either standard (11) or were those recommended by the manufacturer. Shrimp alkaline phosphatase was purchased from Ambion (Austin, TX). Restriction enzymes were purchased from U.S. Biochemicals (Amersham, Arlington Heights, IL) and Promega Corporation (Gaithersburg, MA). Reagents for the preparation of blunt-ended *ERG6* fragment and ligation reactions were purchased as kits from Panvera Corporation (Madison, WI). Dideoxynucleotide sequencing of plasmid DNA clones was performed by the chain termination method (13) using the reagents and protocol from a Sequenase II kit purchased from U.S. Biochemicals. Oligonucleotides used to prime the sequencing reactions were synthesized at the Texas Tech University Biotechnology Institute Core Facility (Lubbock, TX).

Expression of SMT activity and measurement of enzyme kinetics. Escherichia coli cells that contained pSMTy23 were cultured at 37°C to a culture A_{600} of 0.8 to 1.0. The cells were harvested by centrifugation (4,000 × *g* for 10 min). The resulting pellet was resuspended in 5 mL of buffer (50 mM Tris-HCl, pH 7.5, 2 mM $MgCl_2$, 2 mM 2-mercaptoethanol) and ruptured using a French Press (SLM Instruments, Urbana, IL). The cell debris was removed by centrifugation (12,000 × *g* for 15 min), and the supernatant fraction (batch enzyme) was used as the source of material for the SMT assay system (14). SMT assays were performed on 0.5 mL batch enzyme in reactions that contained 50 μM zymosterol and 50 μM [*methyl*-$^3H_3$]AdoMet as described elsewhere with constant agitation at 55 rpm on a shaker bath at 30°C for 45 min

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Abbreviations: AdoMet, (*S*)-adenosyl-L-methionine; HPLC, high-performance liquid chromatography; ORF, open reading frame; SMT, (*S*)-adenosyl-L-methionine:$\Delta^{24(25)}$-sterol methyl transferase.

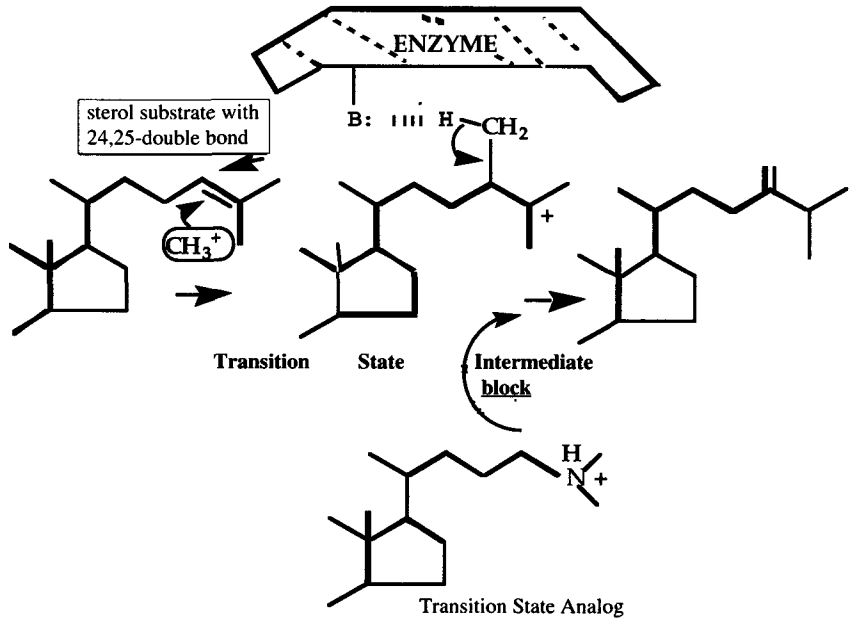

FIG. 1. Mechanism of the (*S*)-adenosyl-L-methionine: $\Delta^{24(25)}$-sterol methyl transferase-catalyzed transmethylation of sterol and (*S*)-adenosyl-L-methionine to yield a 24(28)-methylene sterol. The sterol nomenclature system used in this paper follows the *Nes* rules discussed in Reference 19 and references cited therein. Thus the exocyclic methylene carbons are numbered C-24-C-28, not 24(24').

(15,16). Reactions were terminated by adding 10% methanolic KOH. Nonsaponifiable lipids were extracted from the reaction mixtures with diethyl ether and the organic-soluble sterols and radioactivity analyzed as described (15,16). Inhibitor studies were performed using batch enzyme in the presence of 5 to 100 μM zymosterol and 50 μM [*methyl*-$^3H_3$]AdoMet, and inhibitor concentrations were tested over the range of 1 nM to 100 μM. Inhibition type and K_i values were determined by standard graphical procedures (15,16) for which computer-assisted linear regression analysis afforded

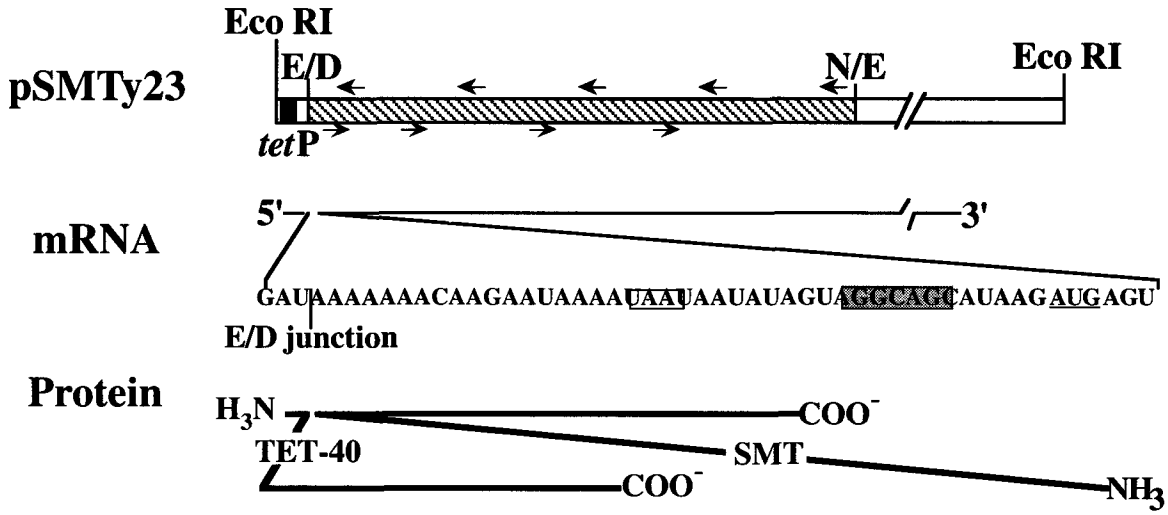

FIG. 2. Cloning, sequencing, and organization of pSMTy23; pSMTy23 DNA is shown as an *Eco*R I linearized plasmid. The *Eco*R V/*Dra* I (E/D) and *Nhe* I/*Eco*R V (N/E) junctions flank the DNA, derived from pRG458, that contains the *ERG6* structural gene (hatched region). The approximate positions and orientation of the oligonucleotide primers used to sequence the pSMTy23 insert are represented by arrows. The pBR322 tetracycline resistance promoter (*tet*P) is represented as a black box. The mRNA that originates from *tet*P has an open reading frame that encodes a truncated (40 amino acid, TET-40) form of the tetracycline resistance gene product whose translation terminates as the result of an in-frame UAA (boxed) located in the cloned DNA fragment. A purine-rich sequence (shaded box), which may serve as a ribosome binding site for (*S*)-adenosyl-L-methionine: $\Delta^{24(25)}$-sterol methyl transferase (SMT) translation, is located six bases upstream of the putative SMT initiator codon (underlined).

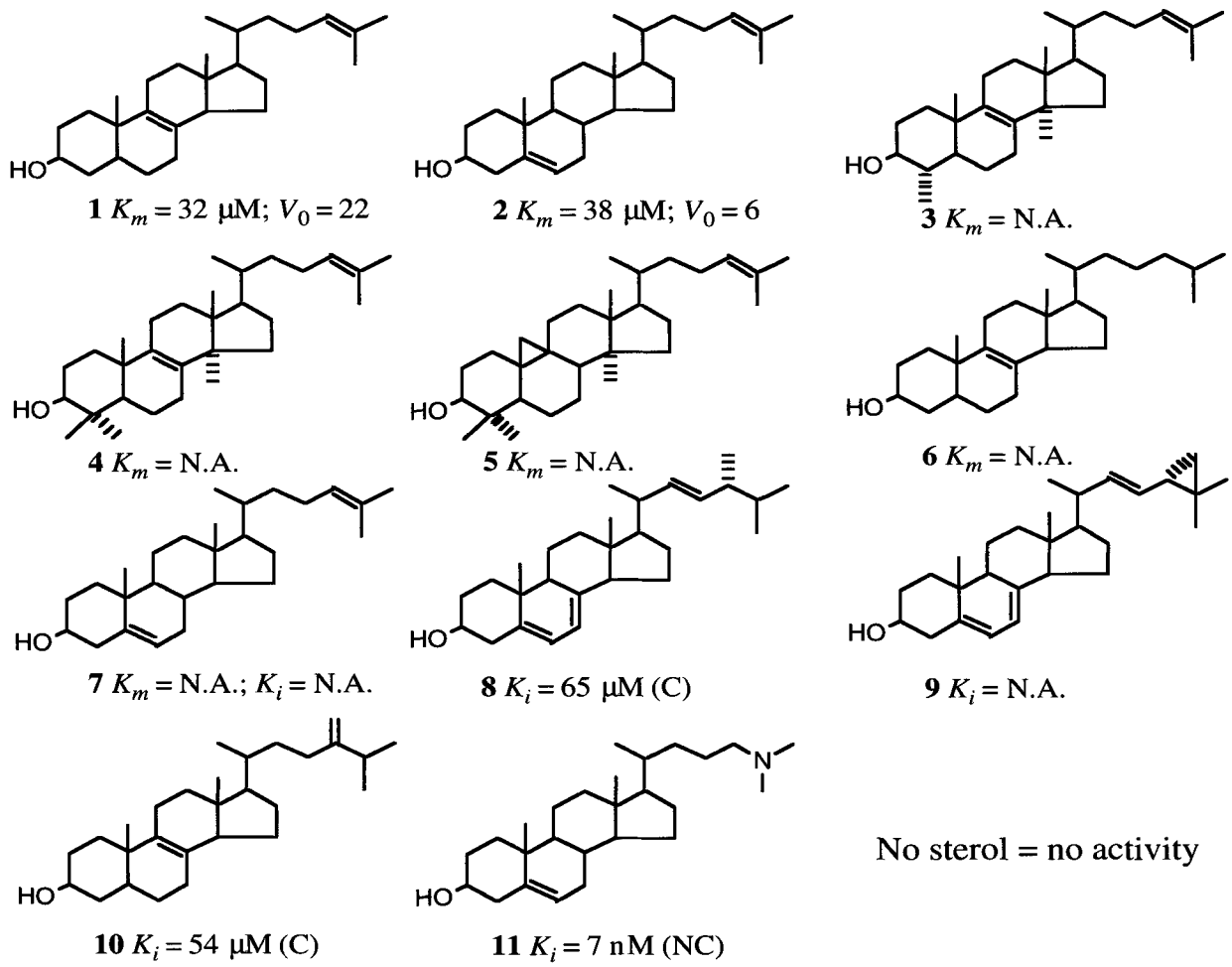

FIG. 3. Sterol substrate specificity and inhibitors of (*S*)-adenosyl-L-methionine: $\Delta^{24(25)}$-sterol methyl transferase enzyme expressed in *Escherichia coli.*

correlation coefficients greater than 0.98 in all trials (n = 3). Products of the SMT reactions were analyzed by high-performance liquid chromatography (HPLC)-radiocounting and gas chromatography/mass spectrometry (GC/MS) (15,16). Protein was determined as described (17). Source of compounds was described elsewhere (15,16; Nes, W.D., and Guo, D., unpublished data). Attempts to overexpress the *ERG6* gene in *E. coli* using plasmid vector, e.g., pLEX (P_L expression system from Invitrogen, San Diego, CA) were impeded by the nonavailability of a restriction site which was appropriate for cloning.

RESULTS AND DISCUSSION

Our initial efforts were directed to the cloning and sequencing of the putative open reading frame (ORF) of *ERG6*. The purpose of these experiments was to confirm that the *ERG6* DNA used in this study was identical to *SED6*, the gene claimed by Hardwick and Pelham (8) to be *ERG6*. *Escherichia coli* has been found to lack the capacity to synthesize sterols (18). For this reason, *E. coli* was chosen as the expression system of the *ERG6* gene. The *SED6* gene sequence published by Hardwick and Pelham (8) was analyzed using the program GeneJockey (Biosoft) to determine that *Dra* 1 and *Nhe* 1 were appropriate restriction enzymes for subcloning the ORF from the plasmid pRG458. The 1.3 kilobase pair fragment obtained by digesting pRG458 with *Dra* 1 and *Nhe* 1 was subcloned into the *Eco*R V site of pBR322. Two unique clonal types, pSMTy23 and pSMTy10, were obtained (Fig. 2). Restriction enzyme digestion patterns of the plasmids determined that these clones differed in the orientation of the insert. DNA sequence analysis of pSMTy23 confirmed that this clone contained a sequence identical to the putative *ERG6* gene published by Hardwick and Pelham (8).

Batch enzyme preparations from cells that contained either pSMTy23 or pSMTy10 were analyzed for SMT activity as described in the Materials and Methods section. Preparations from cells that contained pSMTy23 were found to contain SMT activity, whereas preparations from cells that contained pSMTy10 did not. Our rationale for the basis of SMT

enzyme expression from pSMTy23 in *E. coli* is that *ERG6* mRNA was synthesized in a transcript which originated at the tetracycline resistance determinant promoter of the vector. The bacterial-expressed recombinant protein from this vector system is likely to give rise to the native protein rather than a fusion protein. A purine-rich sequence located upstream of the putative SMT start codon is predicted to serve as a ribosome binding site for translation (Fig. 2).

The functional importance of precise structural and electronic features of the methyl acceptor molecule in supporting SMT activity was first examined in reactions that contained either cholest-8-enol or cholesta-8,24-dienol (zymosterol) as substrate. Methyl transfer occurred only in those reactions that contained zymosterol, demonstrating the importance of the 24,25-double bond in the sterol side chain for catalysis (Fig. 3). HPLC-radiocounting was performed on Whatman reversed-phase C_{18}-columns designed to separate substrate from 24(28)-methylene product and related 24-alkene isomers (19). As shown in Figure 4, zymosterol was stereoselectively biomethylated to fecosterol. To obtain sufficient quantities of biomethylated product for chemical identification, 20 assays were performed, and approximately 4 μg of chromatographically pure sterol was collected from the HPLC column. The sterol was identifed as fecosterol by comparison of its chromatographic behavior in thin-layer chromatography (R_f 0.3, analytical plates developed twice in 85:15 benzene/ether), HPLC (0.77, α_c on Whatman C_{18}-column (Maidstone, England) eluted with MeCN/i-PrOH (9:1) at 1 mL/min at ambient temperature, and gas–liquid chromatography (1.31, retention time relative to cholesterol on 3% SE-30 packed column operated isothermally at 245°C), and by its fragmentation pattern in mass spectroscopy (*m/z*, M^+ 398 amu and other diagnostic ions at 383, 365, 341, 314, 327, 299, 285, 271, 245), with that of an authentic specimen (19).

Several naturally occurring sterols with the 24,25-double bond were tested as substrates; zymosterol and desmosterol were found to function as methyl acceptors in the SMT reaction, whereas lanosterol, cycloartenol, and 31-norlanosterol did not serve as substrates for SMT (Fig. 3). Greater catalytic competence (measured as V_{max}/K_m, 15) was observed with zymosterol than desmosterol. Sterol specificity for SMT catalysis exhibited by the protein expressed from pSMTy23 was similar to that of the native enzyme of *S. cerevisiae* (20,21). In contrast to the plant SMT (15), the yeast sterol-SMT complex was found to be sensitive to steric interference from the C-4 methyl group(s) (20). The isosteric transition state analog for the biomethylation reaction, 24β,25-cyclopropyl ergosta-5,7,(*E*)22-trienol, was not an inhibitor of SMT activity, due to the modification at C-24. However, the isoelectronic transition state analog, 25-azacholesterol (at which physiological pH is protonated) (16) was a potent inhibitor of SMT activity, results consistent with previous reports that 25-azacholesterol and related side-chain ammonium-containing sterols are potent inhibitors of yeast SMT activity (4,5,21).

The SMT from pSMTy23 exhibited a competitive-type inhibition by fecosterol, which is consistent with kinetics from

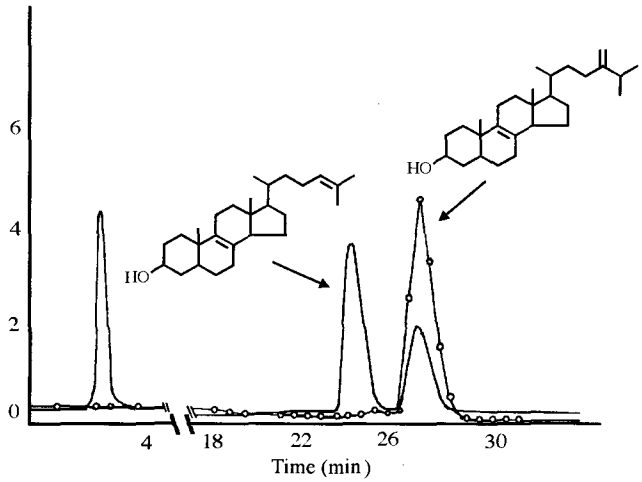

FIG. 4. High-performance liquid chromatography-radiocount chromatogram [Whatman C_{18}-reversed phase column (Maidstone, England) eluted with acetonitrile/isopropanol, 9:1; the ultraviolet detector was set at 205 nm] showing the mass and radioactivity associated with sterols isolated from the thin-layer chromatography-purified nonsaponifiable lipids (NSF) fraction. The NSF was diluted with 50 μg of nonradioactive fecosterol. The sample was obtained from reactions that contained zymosterol and [$^3H_3$-methyl](*S*)-adenosyl-L-methionone as substrates and batch enzyme prepared from pSMTy23-containing cells; O—O, ^3H-radioactivity (dpm x 104); —, detector response (205 nm). Abbreviation as in Figure 2.

product inhibition. When ergosterol and cholesterol were paired as inhibitors, only ergosterol inhibited SMT activity (Fig. 1). This result is expected since cholesterol does not possess a 24-alkyl group. Thus, ergosterol may regulate its synthesis under normal physiological conditions through control of post-lanosterol transformation events as proposed by Moore and Gaylor (20). The inability of others to observe product inhibition by fecosterol (4,5) may be due to the high level of endogenous ergosterol in microsome preparations from wild-type cells (21).

Hydropathy analysis of the SMT enzyme (Fig. 5), based on the predicted amino acid composition from the *ERG6* gene sequence, indicates that the enzyme is moderately hydrophilic with no obvious membrane-localizing elements. For some experiments, batch enzyme was processed by centrifugation at 100,000 × *g* for 90 min to sediment a microsomal fraction (14,22). Microsomal pellets were found to contain 80% of the original SMT activity of the sample, suggesting that the SMT produced in *E. coli* is membrane-associated. Emulphogen-solubilized microsomes released SMT activity with 50% recovery. These observations are consistent with the demonstration that the yeast SMT is loosely bound to the endoplasmic reticulum and distributes into several alternate membrane fractions and into a "floating lipid layer" upon fractionation (14,22).

In this report we demonstrate that the *ERG6* gene expressed from pSMTy23 in *E. coli* produces a protein that mediates biomethylation of zymosterol. The product of the reaction was unambiguously identified as fecosterol using a combination of radiochemical, chromatographic, and spectral

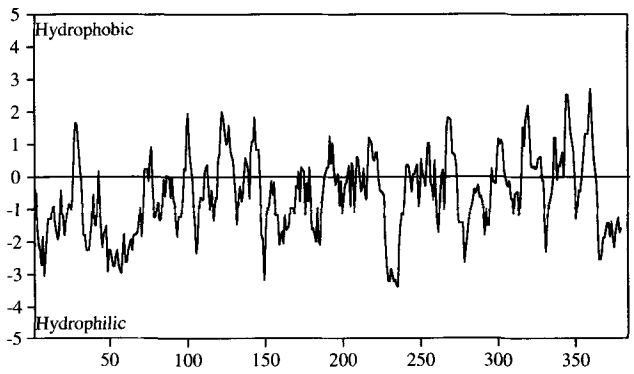

FIG. 5. Hydropathy plot of predicted (*S*)-adenosyl-L-methionine: $\Delta^{24(25)}$-sterol methyl transferase amino acid sequence (Ref. 8).

methods. Down-regulation of the SMT enzyme activity from pSMTy23 by select 24-alkyl sterols shows metabolic specificity for the structure of the side chain. These results confirm that *ERG6* is the structural gene for SMT in yeast and that ergosterol may function to regulate its own synthesis under normal physiological conditions in yeast.

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