GRAPHICAL ABSTRACT

Characterization, Mutagenesis and Mechanistic Analysis of an Ancient Algal Sterol C24- Methyltransferase: Implications for Understanding Sterol Evolution in the Green Lineage

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Cloned sterol C24-methyltransferase from *Chlamydomonas reinhardtii* generates multiple products and possesses broad substrate specificity. The C24- methylation pathway proceeds through the $\Delta^{25(27)}$ -olefin route which furnishes the 24 β -methyl group in algal ergosterol.

**Characterization, Mutagenesis and Mechanistic Analysis of an Ancient Algal Sterol
C24- Methyltransferase: Implications for Understanding Sterol Evolution in the
Green Lineage**

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Abbreviations: SMT, Sterol C24-methyltransferase; SAM, S-adenosyl-L-methionine;
GC-MS, Gas chromatography-mass spectroscopy

Abstract

Sterol C24-methyltransferases (SMT) constitute a group of sequence-related proteins that catalyze the pattern of sterol diversity across eukaryotic kingdoms. The only gene for sterol alkylation in green algae was identified and the corresponding catalyst from *Chlamydomonas reinhardtii* (Cr) was characterized kinetically and for product distributions. The properties of CrSMT were similar to those predicted for an ancient SMT expected to possess broad C3-anchoring requirements for substrate binding and formation of 24 β -methyl/ethyl $\Delta^{25(27)}$ -olefin products typical of primitive organisms. Unnatural $\Delta^{24(25)}$ -sterol substrates, missing a C4 β -angular methyl group involved with binding orientation, convert to product ratios in favor of $\Delta^{24(28)}$ -products. Remodeling the active site to alter the electronics of Try110 (to Leu) results in delayed timing of the hydride migration from methyl attack of the Δ^{24} -bond, that thereby produces metabolic switching of product ratios in favor of $\Delta^{25(27)}$ -olefins or impairs the second C₁-transfer activity. Incubation of [27-¹³C]lanosterol or [*methyl*-²H₃]SAM as co-substrates revealed the CrSMT catalyzes a sterol methylation pathway by the “algal” $\Delta^{25(27)}$ -olefin route, methylation proceeds by a conserved S_N2 reaction and deprotonation proceeds from the pro-Z methyl group on lanosterol corresponding to C27. This previously unrecognized catalytic competence for an enzyme of sterol biosynthesis and phylogenomic analyses suggest that mutational divergence of a promiscuous SMT produced substrate- and phyla-specific SMT1 (catalyzes first biomethylation) and SMT2 (catalyzes second biomethylation) isoforms in red and green algae, respectively, and in the case of SMT2 selection afforded modification in reaction channeling necessary for the switch in

ergosterol (24 β -methyl) biosynthesis to stigmasterol (24 α -ethyl) biosynthesis during the course of land plant evolution.

Keywords: green algae, sterol evolution, sterol C24-methyltransferase, ergosterol, cholesterol, SMT2, SMT1

1. INTRODUCTION

Membrane-bound C₂₈-ergosta- and C₂₉-stigmasta-type sterols of different C24-stereochemistry have contributed to the evolution of primary metabolism in eukaryotes through biosynthetic pathways that can differ significantly (Nes, 2011; Volkman, 2005; Weete et al., 2010). The uneven distribution of fossil C₂₈- and C₂₉-steranes that date to the Precambrian (Brocks et al, 1999; Love et al, 2009; Summons et al., 2006) and phylogenomic analysis of genes of sterol biosynthesis (Desmond and Gribaldo, 2009) suggest that all the necessary enzymes for the formation of phytosterols (24-alkyl sterols), as well as cholesterol, may have existed in a last eukaryotic common ancestor. In the biosynthesis of ergosterol **3** from glucose via the acetate-mevalonate or mevalonate-independent pathways, it is now evident that quite separate, independent molecular evolution occurred in fungi and green algae (Lichtenthaler, 1999; Lombard and Moreira, 2011). However, evidence also is available showing land plants can generate stigmasterol **4** by the same acetate-mevalonate pathways as used by fungi (Miller et al, 2012; Opitz et al, 2014) (Figure 1). Based on the divergent structures of a 24 β -methyl group and 24 α -ethyl group in the final products, one might expect that functional differences in sterol methylating enzymes, sterol C24 methyltransferases (SMTs) responsible for biomethylation along the sterol side chain, would be phylogenetically significant, perhaps even crucial to generation of the panoply of sterol patterns observed throughout nature.

The family of SMTs is considered to be a group of homologous enzymes derived from a common ancestor and are therefore structurally related (Nes, 2000). These slow-acting catalysts show a high degree of sequence similarity, possess tetrameric subunit organization and use comparable mechanistic features to carry out the C24-methylation

reactions (Nes et al, 1998; Nes et al, 2003; Zhou et al, 2006). Testing variant acceptor molecules of heterologously expressed enzymes across kingdoms indicates that substrate specificities evolved differently in the two major classes of SMTs identified in the GenBank as SMT1 and SMT2. All SMT enzymes from fungi and plants accept $\Delta^{24(25)}$ – substrates; the fungal SMT1 that prefers zymosterol **9** is given the designation EC 2.1.1.41, whereas the land plant SMT1 and SMT2 that prefer cycloartenol **2** and 24(28)-methylene lophenol **17** are given the designation EC 2.1.1.142 and EC 2.1.1.143, respectively (Benveniste 2004; Zhou and Nes 2003).

Fungi and green algae SMTs can convert protosterol intermediates to 24 β -methyl sterols by convergent C24-methylation pathways commencing with the transfer of the electrophilic S-methyl group of SAM to C24 of a Δ^{24} -sterol precursor (Nes 2011). The cations produced can be eliminated through a phyla-specific deprotonation at C28 or C27 affording $\Delta^{24(28)}$ -olefins **e** in fungi or $\Delta^{25(27)}$ -olefins **d** in green algae, respectively (Figure 2; Supplementary Figure S1). On the other hand, the $\Delta^{24(28)}$ -route is expressed in the biosynthesis of 24 α -ethyl sterols through the successive action of SMT1 and SMT2 (Bouvier et al 2005; Neelakandan et al 2009), suggesting a recapitulation of the fungal C24-methylation pathway through SMT1 that diverged to produce SMT2. Equally intriguing is the possibility based on bioinformatics analyses of amino acid sequences of SMTs annotated in the GenBank that the genome of green algae may be unique amongst primitive organisms and contain a single SMT2 gene bifunctional in substrate recognition (Supplementary Figure S2 and Table S1). This gene may have originated from a promiscuous SMT of the last eukaryotic common ancestor very early in the evolution of plants, and then diverged to yield SMT1 and SMT2 of land plants.

These opposing views for how SMTs evolved in distantly related groups led us to investigate the catalytic strategy of an early stage SMT at the root of the green lineage. Completion of the *Chlamydomonas reinhardtii* (*Cr*) genome project in green algae revealed that a SMT in the ergosterol biosynthesis (basal trait) pathway with homology to land plant SMT2 implicated in stigmasterol biosynthesis (derived trait) might be present in this primitive organism. Here, we present functional characterization and mechanistic analysis of cloned *Cr*SMT and product analysis of a remodeled active site from site-directed mutagenesis of a catalytically relevant Tyr¹¹⁰ (= Tyr⁸¹ of Erg6p) residue (Nes et al, 2002). By defining the overall rate enhancements and product specificities that distinguish catalytic behavior of an algal SMT from those of more-advanced SMT forms may provide insights into the evolutionary forces that determine the different side chain constructions of phytosterols in plants and fungi and why SMT2s, which often mediate the balance of C24-methyl/ethyl sterols in plants (Schaller et al, 2000; Schaller et al 2001), appear to be restricted to the photosynthetic lineage.

2. RESULTS

2.1 Functional expression of *CrSMT*

Using protocols previously successful to provide functional confirmation of the identity of a SMT gene in bacterial expression studies (Nes et al, 1998), we incubated *CrSMT* first with cycloartenol because this compound is considered the initial protosterol intermediate for ergosterol biosynthesis in *C. reinhardtii* (Nes, 2011; Summons et al. 2006). Activity assays performed under initial velocity conditions afforded approximate K_m and V_{max} values for cycloartenol of $35 \mu\text{M} \pm 3$ and $100 \text{ pmol/min/mg protein}$, respectively (Supplementary Figure S3), similar to the catalytic competency of other SMTs incubated with the corresponding natural substrate. Other properties of the *CrSMT* such as pH optima (between 7.0 and 8.0) and temperature optima (between 30 and 35°C) were similar to those reported previously for 24-SMTs [Nes et al, 1998, Nes et al, 2003; Zhou et al, 2006; Zhou and Nes 2003]. The conversion rate was linear with increasing time up to 90 minutes in a total lysate protein concentration of 1-2 mg/mL.

Product distribution by GC-MS analysis of the cycloartenol incubation with *CrSMT* revealed a mixture of three 24-alkyl(idene) sterols, formed in 70 % overall yield in a ratio of 55:43:2 (Figure 3A). From a preparative incubation with cycloartenol individual 24-alkylated olefins were isolated by HPLC and the identity of each product verified from comparison with authentic standards as 24 β -methyl cycloart-25(27)-enol (cycloaudenol) **7** (product of the first biomethylation); ((EI-MS m/z [rel. int. (relative intensity)]): 440 (8) [M^+ (molecular ion)], 425 (17) [M^+ - methyl], 422 (20) [M^+ - H_2O], 407 (37) [M^+ - methyl - H_2O], 379 (21) [M^+ - propyl - H_2O], 353 (10) [M^+ - part of A ring - H], 315 (7) [M^+ - SC], 300 (32), 95 (100), 83 (31), 69 (92) [C-24–C-27 allylic cation⁺],

55 (70); δ_{H} : 4.667 (H₂-27, brd), 1.640 (H₃-26, s), 0.999 (H₃-28, d, *J* 7.0), 0.957 (H₆-18, s), 0.859 (H₃-21, d, *J* 6.3), 0.550 (H₁-19 *endo*, d, *J* 4.1), 0.330 (H₁-19 *exo*, d, *J* 4.1)), 24(28)-methylene cycloartanol **14** (second product of the first biomethylation) (440 (7) [M⁺ (molecular ion)], 425 (17) [M⁺ – methyl], 422 (23) [M⁺ – H₂O], 407 (40) [M⁺ – methyl – H₂O], 379 (21) [M⁺ – propyl – H₂O], 353 (7) [M⁺ – part of A ring–H], 315 (5) [M⁺ – SC], 300 (27), 95 (100), 83 (40) [C-23–C-27 allylic cation⁺], 69 (94), 55 (87); δ_{H} : 4.720 (H₁-28, brs), 4.670 (H₁-28, brs), 1.031 (H₃-27, d, *J* 6.9), 1.026 (H₃-26, d, *J* 6.8), 0.968 (H₆-18, 30, s), 0.896 (H₃-21, d, *J* 6.5), 0.555 (H₁-19 *endo*, d, *J* 4.2), 0.334 (H₁-19 *exo*, d, *J* 4.2)), and 24 β -ethyl cycloart-25(27)-enol (product of the second biomethylation) (454 (8) [M⁺ (molecular ion)], 439 (16) [M⁺ – methyl], 436 (18) [M⁺ – H₂O], 421 (31) [M⁺ – methyl – H₂O], 393 (17) [M⁺ – propyl – H₂O], 367 (8) [M⁺ – part of A ring – H], 314 (26) [M⁺ – SC – H], 95 (88), 83 (42) [C-23–C-27 allylic cation⁺], 69 (69), 55 (100); δ_{H} : 4.730 (H₁-27, brd), 4.646 (H₁-27, brd), 1.612 (H₃-26, t, *J* 7.0), 0.965 (H₃-18, s), 0.849 (H₃-21, d, *J* 6.5), 0.808 (H₁-29, t, *J* 7.5), 0.549 (H₁-19 *endo*, d, *J* 4.0), 0.327 (H₁-19 *exo*, d, *J* 4.0)), respectively. The chromatographic and spectral analysis of the enzyme-generated sterol mixture clearly shows *CrSMT* can produce multiple 24-methyl/ethyl products from the successive methylation of a Δ^{24} -substrate. These results, together with *in vivo* labeling studies (Miller et al, 2012), demonstrate methylation of the side chains of ergosterol (24 β -methyl) and 7-dehydroporiferasterol **5** (24 β -ethyl) of green algae of the class Chlorococcales can be carried out by a single enzyme. In contrast, land plants utilize separate SMT1 and SMT2 enzymes in the methylation of the side chains of campesterol (24 α -methyl) and stigmasterol (24 α -ethyl), respectively.

2.2 Sequence of *CrSMT*

The *CrSMT* cDNA expressed in *E. coli* generated a polypeptide with an open reading frame of 1,161 bp that encodes for an apparent protein of 387 amino acids, which included Tyr¹¹⁰ (= Tyr⁸¹, *ScSMT*) and 62 other amino acids (16 % of total residues) conserved in the primary structure. The four substrate binding segments typical of SMTs were arranged in the same order and separated by comparable intervals along the polypeptide side chain (Fig. 4). Additionally, the residues found in the catalytic C-domain forming an α/β -Rossmann fold in the core of nucleotide binding proteins and SAM-dependent methyltransferases were also present in this class of SMT (Liscombe et al, 2012). The deduced amino acid sequence of *CrSMT* deposited in the GenBank matched our experimentally determined sequence and shows greater homology to known land plant SMT2s (48 % similarity) than to known SMT1 (34 % similarity) from fungi or protozoa (Supplementary Figure S4). As noted before, phylogenetic relationships of over 70 SMTs using alignments based on the CLUSTAL X program with standard settings for protein alignment show the *CrSMT* to cluster in the SMT2 protein family typically assigned to land plants (Supplementary Figure S2 and Table S1) (Zhou et al, 2008).

2.3 Substrate acceptance for *CrSMT* catalysis

The influence of substrate structure on *CrSMT* sterol binding has been determined by using a series of substrates that differ in a single molecular feature or a combination of similar features. To evaluate the optimal substrate of *CrSMT* was incubated first with potential acceptor molecules synthesized by *C. reinhardtii*, cycloartenol, obtusifoliol **16**, 24(28)-methylene cycloartanol, cyclolaudenol and ergosta-5,7,25(27)-trienol **13**, then incubated with unnatural substrates considered preferred

substrates of SMT1 and SMT2 of fungi and land plants, including zymosterol or lanosterol **1** and 24(28)-methylene lophenol, respectively. Initial steady-state kinetic analyses revealed the algal SMT can differentially recognize the test substrates within a relatively narrow range of K_m values from 18 μM for 24(28)-methylene lophenol to 25 μM for cycloartenol, or there is no productive binding of the $\Delta^{25(27)}$ -substrates ergosta-5,7,25(27)-trienol and cyclolaudenol (Table 1). A noticeable correlation between production rate (V_{max}) and loss of substrate-active site complementarity is observed by modifications to the Δ^{24} -substrate double bond undergoing alkylation. Thus, optimal binding interactions occurred with $\Delta^{24(25)}$ -sterols that mimicked the structure of cycloartenol (V_{max}/K_m of 5.5 = 100% activity) and approximately 50-70 % less activity for $\Delta^{24(28)}$ -sterol substrates that mimicked the structure of obtusifoliol.

These results for the isolated enzyme are consistent with *in vivo* studies (Miller et al, 2012) showing the natural substrates of the first and second C₁-transfer reactions are cycloartenol and obtusifoliol, respectively (*cf.* biosynthesis pathway in Supplementary Figure S1). The inability of CrSMT to metabolize 24 β -methyl $\Delta^{25(27)}$ -sterols which is necessary to further alkylate the sterol side chain of many uncommon triply alkylated sterols reported in marine organisms (Kerr and Baker, 1991) shows the progression of sterol methylation is limited in the green lineage. Alternatively, three acceptor molecules not synthesized by *C. reinhardtii* (lanosterol, zymosterol and 24(28)-methylene lophenol) are recognized with equal efficacy to the endogenous substrates cycloartenol or obtusifoliol which suggests a certain degree of substrate promiscuity in green algae which is not found in fungi. These unusual observations prompted further study of the product specificities of CrSMT by GC-MS analysis.

2.4 Variation in product specificity

The product outcomes of 17 acceptor molecules, incubated as before, can separate into two groups based on the side chain olefin structure as Group I- $\Delta^{24(25)}$ or Group II- $\Delta^{24(28)}$ (Figure 5). In the absence of competing $\Delta^{24(25)}$ -substrate, substrates of Group II convert in relatively high yield to a single 24-ethyl product, typified by the conversion of 24(28)-methylene cycloartanol to 24-ethyl cycloart-25(27)-enol (Figure 3, Panel B). A single product is formed whether the $\Delta^{24(28)}$ -substrate contains zero, 1 or 2 methyl groups at C4 in the nucleus. To establish the generality of the 24 β -ethyl stereochemistry in the C₂-transfer product, we incubated obtusifoliol with CrSMT and purified the product by HPLC. The isolated product afforded: HPLC retention time of α_c 1.2; EI-MS (m/z [rel. int. (relative intensity)]) 440 (42) [M^+ (molecular ion)], 425 (100) [M^+ - methyl], 422 (2) [M^+ - H₂O], 407 (18) [M^+ - methyl - H₂O], 341 (7) [M^+ - part of A ring + H], 313 (4) [M^+ - A ring], 300 (4) [M^+ - SC - H], 95 (43), 83 (57) [C-23–C-27 allylic cation⁺], 69 (42), 55 (66); δ_H : 4.739 (H₁-27, dd, J 2.0, 2.5), 4.653 (H₁-27, d, J 2.5), 1.573 (H₃-26, s), 0.974 (H₃-19, s), 0.890 (H₃-21, d, J 6.5) 0.707 (H₃-18, s), 0.810 (H₃-29, t, J 7.2) (Supplementary Figure S5). The NMR assignments of the isolated sterol are consistent with the product side chain bearing the 24 β -ethyl stereochemistry and a $\Delta^{25(27)}$ -double bond (Goad and Akihisa, 1997).

Retention of the $\Delta^{25(27)}$ -double bond was confirmed in the product of the second alkylation reaction of the substrate 24-methyl cycloart-24(25)-enol (cyclobranol) **6** by a combination of chromatographic and spectral analysis (Supplementary Figure S6): HPLC retention time of α_c 1.9; EI-MS, 454 (4) [M^+ (molecular ion)], 439 (7) [M^+ - methyl], 436 (9) [M^+ - H₂O], 421 (16) [M^+ - methyl - H₂O], 393 (8) [M^+ - propyl - H₂O], 367 (4) [M^+ -

part of A ring - H], 314 (16) [M^+ -SC - H], 95 (100), 83 (91) [C-23–C-27 allylic cation⁺], 69 (73), 55 (98); δ_H : 4.722 (H₂-27, brd), 4.661 (H₂-27, brd), 1.607 (H₃ - 26, t, *J* 11.0), 0.966 (H₆-18,30, s), 0.861 (H₃-21, s), 0.808 (H₆-28,28', s), 0.549 (H₁-19 *endo*, d, *J* 4.5), 0.325 (H₁-19 *exo*, d, *J* 4.5). This unnatural CrSMT-generated product cycloneolitsol contains an uncommon dimethyl group at C24 and is occasionally detected in marine organisms and protozoa (Goad and Akihisa, 1997). Alternatively, incubation of cyclobranol with land plant SMT1 from soybean yields the 24(28)-methylene 25-methyl side chain (Wang and Nes, 2008).

However, unexpected and unprecedented product ratios were observed from incubation of $\Delta^{24(25)}$ -substrates that possess a different C4-atom substitution from cycloartenol that contains a 4,4-dimethyl group. Thus, $\Delta^{24(25)}$ -substrates that contain a geminal C4-methyl group convert to $\Delta^{25(27)}$ - and $\Delta^{24(28)}$ -olefin product ratios of approximately 55:43, whereas substrates with no C4 β -angular methyl group, as in 31-nor-lanosterol **8**, convert to a product ratio of 38:57 and substrates with no C4-methyl groups, as in zymosterol, convert to a product ratio of 24:72 (Figure 3, Panel C). This remarkable metabolic switching in product ratios likely result from changes in C4-steric effects on the polarity of the C3-anchor group, that therefore can posit the terminal methyl groups of the bound intermediate somewhat further away (as for C27) or closer (as for C28) to the deprotonating base (Supplementary Figure S7). Although the catalytic proficiencies for alternative substrates tested with CrSMT could be similar for natural and unnatural substrates, no substantial rate enhancements for $\Delta^{24(28)}$ -substrates over the $\Delta^{24(25)}$ -substrates are achieved. This contrasts with the situation of SMT2 from land plants

where rate enhancements are detected for select $\Delta^{24(28)}$ -substrates (e.g., 24(28)-methylene lophenol) over $\Delta^{24(25)}$ -substrates (e.g., cycloartenol).

2.5 Mechanistic analysis

Early studies have established two parallel C24-alkylation pathways for the synthesis of 24 β - and 24 α -isomers in phytosterol biosynthesis across kingdoms (Goad and Akihisa, 1997; Kerr and Baker, 1991). The key to this chemodiversity originates in the stereochemical path and regiospecificity of the coupled-methylation-deprotonation reaction. It is well established that the 24-methyl and 24-ethyl groups (C28 and C29) in ergosterol and 7-dehydroporiferasterol produced by green algae when grown in presence of [*methyl*- $^2\text{H}_3 = \text{CD}_3$]methionine, should contain up to three and five deuterium atoms in the molecules, respectively (Miller et al, 2012; Nes, 1977). In contrast, campesterol and stigmasterol incorporate 2 and 4 deuterium atoms in the 24-alkyl group, respectively (Nes, 1977). Therefore, it was not surprising to observe that cloned *Cr*SMT incubated with [*methyl*- $^2\text{H}_3$]SAM and lanosterol afforded 24-methyl(ene)-products that incorporated three and two deuterium atoms ($\text{M}^+ 443$ and $\text{M}^+ 442$, respectively; Table 2). GC-MS analysis of the sterol mixture revealed a notable change in the proportion of olefin products in the direction of 24 β -methyl lanosta-8,25(27)-dienol. This shows the operation of a pronounced kinetic isotope effect (KIE) during the stabilization of C25 cation **b** and indicates that proton loss from C28 **c** is the rate-limiting step in $\Delta^{24(28)}$ -sterol formation (Figure 2). These data further support our recent conclusion using cloned soybean SMT1 that the coupled methylation-deprotonation pathway affording multiple products has a fast kinetic step as the alkylation of the Δ^{24} -double bond to give initially a

C25-carbonium ion and a slow deprotonation step that establishes the product specificity (Patkar et al, 2013).

For the side chain formation of ergosterol and 7-dehydroporiferasterol in green algae, a C24-methylation reaction mechanism that involves β -face attack of the “methylation” on the Δ^{24} -substrate bond has been proposed (Figure 2) (Nes 2011). Based on the product specificity of CrSMT reported above, an S_N2 -type SAM bioalkylation is demonstrated through ^1H NMR identification of the 24 β -alkyl group stereochemistry for enzyme-generated products 24 β -methyl cycloart-25(27)-enol and 24 β -ethyl cycloart-25(27)-enol, respectively. Mass spectral data of the three enzyme-generated products show a one mass unit increase over the corresponding non-labeled product (Table 2). The molecular ion clusters for ^2H [D]-labeled 24(28)-methylene product (M^+ 441) show increased peak intensities in the high mass end region from loss of isopropyl group at M^+ -44 (m/z 397) and scission between C22-C23 of m/z 84 at m/z 357, thus revealing the presence of deuterium label at C25. Alternatively, the enhanced fragment ion for allylic cleavage through the C22-23 bond at M^+ - 70 (m/z 371) and fragment ion of m/z 84 in the deuterium labeled 24-methyl/ethyl $\Delta^{25(27)}$ -products (M^+ 441 and M^+ 455 , respectively) show the ^2H -atom is retained at C24 in these molecules (Supplementary Figures S8-S10).

^{13}C NMR analysis of the two diastereotopic methyl groups at C25 in the ^{13}C -labeled product show the signal for pro-*R* methyl group (C27) (Dennis and Nes, 2002, Guo et al, 1996) is markedly enhanced at 21.86 ppm (Figure 6). The general picture which emerges from these studies is one in which the Pro-*Z* methyl group on cycloartenol corresponding to C27 is transformed into the Pro-*R* methyl group at C25 on 24(28)-methylenecycloartanol by migration of the hydrogen atom at C24 to C25 from the *Re*-

face of the substrate double bond (Figure 2). ^{13}C NMR analysis of the other enzyme-generated products from incubation of $[27\text{-}^{13}\text{C}]$ lanosterol ($[27\text{-}^{13}\text{C}]\mathbf{1}$) or $[27\text{-}^{13}\text{C}]$ eburicol ($[27\text{-}^{13}\text{C}]\mathbf{15}$) (recovered from the sterol mixture of the $[27\text{-}^{13}\text{C}]$ lanosterol) show a single enhanced peak resonating at 109 ppm (Figure 6). These observations indicate the biosynthetic inequality of chemically equivalent C26 and C27 and for regiospecific proton elimination ($\text{CH}_3 \rightarrow \text{CH}_2$) at the *cis* terminal methyl group of the substrate double bond to become C27. The absence of scrambling of methyl groups during the biosynthesis of 24-methyl or 24-ethyl sterols as can occur in the biosynthesis of some pentacyclic triterpenoid (Kushiro et al, 1999) indicates the isopropyl group of the 24β -alkyl cation must be held tightly by CrSMT.

2.6 Remodeling the CrSMT active site at position-110

A combination of sequence alignments, molecular modeling and site-directed mutagenesis experiments were used previously to identify residues that contribute to catalytic specificity within the substrate recognition segment of Region I, considered the signature motif for this class of catalyst (Ganapathy et al, 2008). Recent studies focused on modification of the enzyme active site by site-directed mutagenesis revealed the aromatic amino acid at position-81 (tyrosine) in Erg6p is essential for the successive methylation of the Δ^{24} -bond (Ganapathy et al, 2008). We also discovered this residue functions mechanistically in the Michaelis complex to stabilize the bound intermediate through cation- π interactions (Nes et al, 2008). Additionally, our data on Erg6p inhibition reveal ground-modeled inhibitors have little or no effect in binding due to Tyr⁸¹ mutation whereas transition-state inhibitor binding is greatly affected because of the substitution on Tyr⁸¹ (Nes et al, 2008). This observation is consistent with the role of the Tyr⁸¹ residue

during transition-state stabilization, while it may not be required for substrate binding. Taking into account these catalysis, two site-specific mutations targeted at the Tyr¹¹⁰ residue in *Cr*SMT (= Tyr⁸¹ in Erg6p; *Sc*SMT) were constructed to inspect the function of the polar residue. Residue numbers described hereafter correspond to the consecutive numbering of the full-length amino acid sequence of a given gene beginning with the start codon. For example, using Erg6p as reference the amino acid numbers in *Cr*SMT are frame shifted up by 29 amino acids whereas for *Tb*SMT the residues are shifted down by 15 amino acids (Supplementary Figure S4).

GC-MS analysis of enzyme-generated products from the Phe mutant versus wild-type enzyme showed that removal of the OH group from tyrosine had little effect on catalysis (Table 3), suggesting a change in bulk at position-81 can be tolerated. These results were disappointing since in related studies of fungal and plant SMT1, the Tyr→Phe mutation afforded a gain-in-function such that the mutant Erg6p proceeds to carry out a plant-like second biomethylation of fecosterol **18** (Kushiro et al, 1999), or the mutation can in soybean enzyme produce unusual product ratios from biomethylation of 24(28)-methylene cycloartanol (Nes et al, 2006). On the other hand, substitution of Tyr81 by leucine, an aliphatic residue that can alter the polarity of the contact amino acid involved with stabilization of the bound intermediate cation, yielded a mutant enzyme that converts cycloartenol and zymosterol to product ratios that favor the formation of $\Delta^{25(27)}$ - olefins (Figure 2, Panel C), or as shown by incubation of 24(28)-methylene cycloartanol the second C₁-transfer activity is inhibited. In most cases where the C25 cation is formed by attack of “CH₃⁺” on the Δ^{24} -bond, several alternatives exist for stabilization (Figure 2). The one that is perhaps most expected, if the process is

concerted, is removal of a proton from one of the gem-dimethyl groups, that thereby eliminates the tertiary cation (i.e., to form cycloaudenol). However, for this outcome to take place at the expense of $\Delta^{24(28)}$ -formation, as can occur during the second C₁-transfer reaction, there must be proper positioning of a stabilizing residue to control the timing of the 1,2-hydride shift involved with elimination of the C24-cation. In particular, the secondary carbocations are significantly farther away to the deprotonating base than the preceding tertiary carbocations (Figure 2). Accordingly, we hypothesize that the introduced aliphatic residue in *CrSMT* at position-110, which is inert, fails to adequately stabilize the C24-cation such that the ensuing reaction channeling during the course of biomethylation is interrupted.

3. DISCUSSION

The current studies were undertaken, in part, to define further enzyme action responsible for the expansion of chemodiversity in phytosterol biosynthesis, and furthermore to probe in depth the fundamental properties of SMT enzymes that led to the switch in C24-methylation routes from producing algal ergosterol and 7-dehydroporiferasterol (24 β -ethyl group) to producing land plant stigmasterol (24 α -ethyl group). A simplified overview of phytosterol biosynthesis with SMT as a biomarker of the phyla-specific pathways is shown as Supplementary Figure S1. The class of Chlorophyceae within the Kingdom of Viridiplantae has been placed at the bottom of the eukaryotic tree (Cavalier-Smith, 2004). In the present report, we show green algae encode a bifunctional SMT2 that catalyzes 24 β -methyl/ethyl sterol products that strongly supports this theory. Our phylogenetic analysis shows that SMT2 and SMT1 lineages evolved repeatedly from a common ancestral gene, likely concurrent with the origin of the eukaryote domain, and then lost variably in some lineages because no SMT homologs from prokaryotes or advanced animals (above the sponges) were identified (Figure 7). It is interesting that plant-derived SMT genes acquired different catalytic strategies upon mutational divergence into SMT1 (red algae) and SMT2 (green algae). The tendency for preferential binding of cycloartenol to plant SMT1 and zymosterol to fungal SMT1 compared to the preferential binding of 24(28)-methylene lophenol to land plant SMT2 support the notion these enzymes evolved their requirements for C4-substituted substrates independently. Given that land plant SMT1 possesses a vestigial $\Delta^{25(27)}$ -olefin pathway (Patkar et al, 2013) and that green algal SMT2 possess a binding pocket for 24(28)-methylene lophenol suggest an algal-like SMT2 underwent mutational divergence

affording separate land plant SMT1 and SMT2 isoforms; these enzymes acquired the $\Delta^{24(28)}$ -route to form 24Z-ethylidene sterols (Benveniste, 2004; Bouvier et al, 2005; Neelakandan et al, 2009) required in the synthesis of 24 α -alkyl sterols. In this way, SMT2 evolution has remained confined to the green lineage.

In pursuing our discussion of SMT evolution we have taken an empirical approach which emphasizes a major determinant of molecular diversity in sterol side chain structure is the precise anchoring system of ring-A and the conformation of the bound intermediate at the active site (Howard et al, 2012). Comparative analysis with the genomes of plants, fungi and protozoa indicates that the SMT genes involved with phytosterol biosynthesis are orthologous among these organisms. Accordingly, these gene products should display functional equivalence with increases in catalytic efficiency and improvements in product ratios resulting from changes in differential binding of substrates associated with the sterol shape and functional groups of the anchoring system (Albery and Knowles, 1977). In this evolutionary scenario, the charge-stabilizing mechanism producing the $\Delta^{24(28)}$ Z-olefin pathway for land plant phytosterol biosynthesis depends on the proximity of the cationic center with the nearest substrate double bond during sterol methylation (Supplementary Figure S7). As we have shown here, the presence of Try¹¹⁰ in *Cr*SMT is relevant to cation- π interactions by stabilizing the ensuing C25- and C24-cations in the intermediate in the active site. The loss of these interactions can lead to directed channeling and loss of the second biomethylation activity; as such this residue functions in algal SMT to control the expansion of the sterol side chain.

SMTs have strongly conserved amino acid sequences and therefore the corresponding common ancestral gene can be envisioned to have possessed a finite number of introns then lost several of them during evolution giving rise to different sets of conserved intron-exon organization for the extant enzymes. A similar proposal has been presented for the evolution of intron-exon organization in RNA sequences of terpene synthases (Lange et al, 2000). Recently, Neelakandan, *et al.* examined the intron-exon structures for 11 SMTs representing green algae, fungi, protozoa and land plants (Neelakandan et al, 2009). Most striking from the genomic organization of SMT from green algae to vascular plants is that these isoforms can be placed into a monophyletic clade and classed by the number of introns- 5 (green algae) to 12 (embryophytes)-dispersed between exons in the SMT structure. On the other hand, land plant SMT2, protozoan SMT1 and some fungal SMT1 are marked by a single conserved intron suggesting that algal SMT2 might have diverged from an ancestral SMT early in plant evolution. Indeed, continued evolution of the green algal SMT2 into the Charophyta could provide the antecedent of land plant SMT1 and SMT2 (Patterson et al, 1991); notably, the Chara synthesizes 24(28)Z-ethylidene cholesterol (isofucosterol) which is an intermediate to stigmasterol biosynthesis (Patterson et al, 1991). Interestingly, red algae typically synthesize cholesterol as their dominant sterol (Beastall et al, 1974; Kodner et al, 2008), and cholesterol is often present in variable amounts in other organisms as diverse as the oomycetous fungi and humans (Nes, 2011; Weete et al., 2010), suggesting a down-regulation or loss of SMT activity from these lineages. Therefore, it is evident that retrograde evolution of SMT could be class-specific, and may have arisen in select lineages due to loss of promoters in the *cis*-sequence motifs located upstream of the

SMT coding sequence (Neelakandan et al, 2010). Thus, directed mutations of regulatory and transcribed regions associated with the SMT gene may be responsible for shifts in sterol biosynthesis pathways which in the lineage generating vertebrate animals led to coupled loss in phytosterols with a gain in cholesterol production.

4. CONCLUDING REMARKS

The results described here provide important clues about the conservation of molecular mechanism versus diversity- creating channel switching in the C24-methylation reaction, especially with regard to the face of the substrate Δ^{24} -bond that is methylated as well the stabilization of the resulting carbocationic intermediate(s) through cation- π interactions. The *CrSMT* relaxed specificity for unnatural substrates and remodeling of the active site through mutagenesis of a catalytically relevant aromatic residue appears not only to provide the framework for the continued expansion and variations in sterol side chain constructions but may also allow further modifications of active site residues by mutagenesis that produce novel sterol profiles affording value-added traits in plants and microorganisms.

5. EXPERIMENTAL

5.1 Chemicals and instrumental analysis

The source of 17 sterol substrates included in this study is described in earlier papers (Miller et al, 2012; Liu et al, 2011; Xu et al, 1988). All sterols were purified by HPLC to <95% by GC analysis. SAM iodide salt was purchased from Sigma, [*methyl*-³H₃]SAM (specific activity 10-15 Ci/mMol and diluted to 10 μ Ci/ μ mol, 1 Ci = 37 GBq) Tetraosylate [*methyl*-²H₃]SAM (SAM is *S*-Adenosyl-L-methionine = AdoMet; 99% atom enrichment) was purchased from C/D/N Isotopes (Pointe-Claire, QC), and [24-²H]lanosterol ([24-²H]**1**) and [27-¹³C]lanosterol (98% atom enrichment) were prepared as previously described (Schaffer et al 2001). Bradford protein assay kit was purchased from Bio-Rad and isopropyl-1-thio- β -D-galactoside (IPTG) was purchased from Research Products International Corp. QuikChange site-directed mutagenesis kit was purchased from Strategene. All other reagents and chemicals were purchased from Sigma or Fisher unless otherwise noted.

Instrumental methods have been reported previously (Liu et al, 2011; Xu et al, 1988). Briefly, proton and carbon NMR spectra were recorded in deuteriochloroform at the indicated frequencies using a Varian Unity Inova 500 MHz spectrometer or JEOL ESC 400 MHz spectrometer. Chemical shifts (δ , ppm) are referenced to TMS (δ , 0). Mass spectra were obtained on a Hewlett-Packard 6890 GC- HP 5973 MSD instrument (electron impact, 70 eV, scan range 50-550 amu). HPLC was carried out using Phenomenex Luna C₁₈-column (250 mm x 4.6 mm x 5 μ m) connected to a diode array multiple wavelength detector (Liu et al, 2011). Capillary GC (0.25 mm i.d. by 30 m fused silica column coated with Zebron ZB-5 from Phenomenex) was operated as previously

described (Miller et al, 2012; Liu et al, 2011). GC and HPLC of sterols is reported as retention times relative to the elution time of cholesterol as RRT_c and α_c , respectively. Product distributions are determined by approximate integration of chromatographic peaks.

5.2 Cloning of full length cDNA of *CrSMT*

By use of conventional, PCR based methods, a full length 1136bp cDNA was assembled from *CrSMT* cDNA clones (1030123H02.y1, 102407H12.y1 and 963068B05.x1) available from the Chlamydomonas Resource Center (<http://chlamycollection.org>) and was identical to the published transcript sequence of *CrSMT* (NCBI: XM_001690723.1 and Merchant et al (2007)). The cDNA was cloned into a pet30a+ (Novagen) between *EcoR* I and *Xho* I sites and transformed into *Escherichia coli* BL21 strain affording a wild-type recombinant *CrSMT* protein of 384 amino acids, three short from the predicted sequence of 387 amino acids. DNASTAR Lasergene 8 sequence analysis programs (DNA STAR Inc,) was used to identify the largest open reading frame of the cDNA sequence of *CrSMT*. The protein sequence of *CrSMT* translated from cDNA was determined in SeqBuilder.

5.3 *CrSMT* assay and product analysis.

For bacterial expression of cloned *CrSMT* protein, a 50 mL culture of *E.coli* BL21(DE3) strain harboring the *CrSMT* cDNA was supplemented with 50 μ L of kanamycin (50 mg/mL) and grown at 30 °C to an optical density (OD) of 0.7. A 20 mL aliquot plus 50 μ L of kanamycin was transferred to 1 L of LB media and incubated at 30 °C for 3 h to an OD of 0.5-0.6 at 225 rpm. Cultures were then induced by the addition of 0.4 mM IPTG and grown for another 2 h to an OD of 1.1-1.3. Cells were harvested by centrifugation

(7500 x g) and re-suspended in 5 mL of phosphate buffer containing 5 % (v/v) glycerol at pH 7.5. The cell pellet was broken by French Press disruption and the resulting lysate preparations, maintained in phosphate buffer throughout, were assayed under initial velocity conditions (1-2 mg total protein) of 45 min using the appropriate sterol acceptor tested over the concentration range 5 to 150 μ M and paired with saturating levels of [methyl- 3 H₃]SAM at 100 μ M (0.6 μ Ci) following standard protocols (Nes et al, 2008). Incubation mixtures were saponified with methanolic KOH and extracted with hexane (3 x 1 mL) to provide the non-saponifiable lipid fraction. This assay involves scintillation counting of the non-saponifiable lipid fraction to determine conversion rate which did not differ by more than 10 %. Incubation of [methyl- 3 H₃]SAM, in the absence of sterol, with enzyme preparation generated background levels of radioactivity between 500 to 1,000 dpm. Data analysis was determined according to the Lineweaver-Burk equation using the computer program Sigmaplot 2001 plus the enzyme kinetics module software package (Nes et al, 2008). In control enzyme preparations of *E. coli*, there is no sterol contaminant detected by GC-MS as would be expected since these compounds are not synthesized by bacteria. Product distributions generated by wild-type and mutant CrSMT were determined by incubating saturating amounts of the co-substrates with sufficiently large preparations of protein (2-3 mg/mL) for 8- 12 h to allow completion of the reaction; products were analyzed by GC-MS or in special cases the products were purified by HPLC and analyzed further by NMR. Product yields determined by capillary GC were accurate to approximately 1 % (50 ng). Protein amounts were quantified by using the Bradford method (Bio-Rad) and bovine albumin as the protein standard.

5.4 Site-directed mutagenesis.

Point mutants, constructed from the wild-type *CrSMT* gene, were made with a pet30a+ vector by using the standard QuikChange mutagenesis method (Stratgene) and confirmed by DNA sequencing. Specific mutagenic primers (sense direction with mutagenic bases shown in boldface) are as follows: Y100F-CTAGTCACT

GACATTT**TC**GAGTGGGGCTGGGGC,Y110LCTAGTCACTGACATT**CT**GAGTG
GGGCTGGGGC. Plasmid DNA constructs were transformed into BL21 (DE3) cells, and expression of the *CrSMT* genes were induced by IPTG addition to the cultures as described for wild-type *CrSMT*. The product distribution generated by mutant SMTs were determined by GC-MS following overnight incubation of saturating amounts of co-substrates (100 μ M) with 3 mg of lysate protein.

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Legend to Figures.

Figure 1. Sterol biosynthesis pathways of phylogenetic significance; AC-MVA is the acetate-mevalonate pathway to Δ^3 -IPP, and MEP is the methyl erythritol-D-phosphate pathway (or MVA-independent pathway) to Δ^3 -IPP.

Figure 2. Alternative C24-alkylation pathways catalyzed by sterol C24-methyltransferase enzymes to $\Delta^{25(27)}$ - or $\Delta^{24(28)}$ -olefin products. ^{13}C -labeled carbon is labeled in green. Stereospecific deprotonation at C28 of Ha-atom or Hb-atom yield the C24(28)*E*-ethylidene sterol or C24(28)*Z*-ethylidene sterol side chain geometry, respectively, as reported in Nes et al, 2003; SN, sterol nucleus.

Figure 3. Total ion current chromatogram of the total sterol fraction recovered from wild-type *CrSMT* preparations incubated with natural and non-preferred substrates. Panel A is incubation of cycloartenol (first peak) followed by enzyme-generated products cyclolaudenol, 24(28)-methylene cycloartanol and 24 β -ethyl cycloart-25(27)-enol; Panel B is incubation of 24(28)-methylene cycloartanol (first peak) followed by enzyme-generated product 24 β -ethyl cycloart-25(27)-enol; and Panel C is incubation of zymosterol (first peak) followed by enzyme-generated products ergosta-8,25(27)-dienol, ergosta-8,24(28)-dienol and 24 β -ethyl ergosta-8,25(27)-dienol, respectively.

Figure 4. Deduced sterol C24-methyltransferase (SMT) amino acid sequence for *Chlamydomonas reihardtii* (GenBankTM accession number, gi|158279461). Identical residues conserved in the primary structure of SMTs studied in this work are blocked in black. Substrate binding segments (boxed) are indicated for sterol- Regions I, III and IV and SAM- Region II.

Figure 5. Product distributions of substrates incubated with the *CrSMT*. Figure is outlined as follows: name of substrate, in parenthesis the systematic name of sterol, percent conversion of substrate to product, in parenthesis catalytic competence normalized to 100% against cycloartenol, and the enzyme-generated product sets of C24-methyl C25(27) olefin to C24(28)-methylene olefin to C24-ethyl C25(27)-olefin.

Figure 6. Partial ^{13}C NMR spectra of ^{13}C -labeled products from incubation of *CrSMT* with [27- ^{13}C]lanosterol (Panel A) or [27- ^{13}C]eburicol (Panel B).

Figure 7. Phylogenetic arrangement of life forms (based on Cavalier-Smith, 2004) correlated to the distribution of SMT types reported in the GenBank. Color schemes for Chromalveolata indicate brown, green and red algae. LECA, last eukaryotic common ancestor. SMTs arranged according to sequence relatedness is reported in Supplementary Figure S2 and Supplementary Table 1.

Figure 1.

A First C₁-Transfer Reaction

B Second C₁-Transfer Reaction

Figure 2.

Figure 3.

RIV
RII
RIII
RI

```

(1)  MA V | A L P A A V T S A Y E R L A G E F D K L S T T Q K Y A V G | I A G G V T S L Y L L A K | V L K G | S D R D K P T T L Q L
(61) S G G | S I D S S K V K D E F T A Y A D S Y G K N A G E C I T D R | S K T V H L V D V F | V S L V | T D I | Y E W C W C Q S F H F
(121) S P K | L P N K D L K A S E A A H E | A R I A A L L R L Q P G Q K A | L D C C C G V G C P M R T | V A A V S G A H I T G I T I N
(181) Q V Q | V D R A K T H N A R Q G L A P L T D V V R G D | F T N M P F K E N T F   G A V A | I E A T C H | A P K L E Q V V G E I Y
(241) R V L | K P G S Y F V S Y E W V S | T Q K F D V N N A E H V K I M D E | I N F G | N G L P E M R T W K E A E D A G K N V C F E L
(301) V M S L | D L A T A S V V A G P W Y E R L R M G K Y T H A I N H G I V S T V D A L G L A P K G | L K E V H H M L V E V A K S
(361) L I Q | C G E S G I F T P M H L L L F R K P G A D K K K

```

Figure 4.

Figure 5.

Figure 6.

Figure 7.

Table 1. Kinetic parameters of sterol substrates assayed with *CrSMT*.

Substrate ¹	K_m (μ M)	V_{max} (pmol/min/mg)	V_{max}/K_m
Cycloartenol	25	137	5.48 (100)
Lanosterol	24	109	4.54 (80)
Zymosterol	22	120	5.45 (99)
24(28)-Methylene lophenol	20	52	2.89 (53)
Obtusifoliol	19	42	2.21 (40)
24(28)-Methylene cycloartanol	18	29	1.61 (29)
Ergosta-5,7,25(27)-trienol	0	0	0
Cyclolaudenol	0	0	0

¹Catalytic competence of cycloartenol conversion to product is normalized to 100 % (in parenthesis). See Figure 5 for a key to structures.

Table 2. Percentage composition of sterol mixtures generated by *CrSMT* incubated with ^2H - or ^{13}C - labeled substrates.

Substrate Pair	Product Ratio ¹ (M^+) ¹		
	1	2	3
Lanosterol/SAM	55 (440)	38 (440)	7 (454)
[27- ^{13}C] Lanosterol/SAM	54 (441)	38 (441)	8 (455)
[24- ^2H]Lanosterol/SAM	54 (441)	38 (441)	8 (455)
Lanosterol/[$^2\text{H}_3$ - <i>methyl</i>]SAM	88 (443)	12 (442)	0 (459)

¹*CrSMT* products determined by GC-MS analysis are: **1**- 24 β -methyl lanosta-8, 25(27)-enol, **2**- 24(28)-methylene lanosterol (eburicol), and **3**, 24 β -ethyl lanosta-8, 25(27)-enol.

Table 3. Product ratios of substrates incubated with wild-type and mutant *CrSMT*.

Strain	Substrate	$\Delta^{25(27)}$ to $\Delta^{24(28)}$ -product ¹ (% conversion)
Wild-type	Cycloartenol	57/43 (70)
	24(28)-Methylene cycloartanol	100/0 (35)
	Zymosterol	28/72 (70)
Tyr110Phe	Cycloartenol	65/35 (45)
	24(28)-Methylene cycloartanol	100/0 (60)
	Zymosterol	30/70 (43)
Tyr100Leu	Cycloartenol	65/35 (10)
	24(28)-Methylene cycloartanol	NC
	Zymosterol	55/45 (55)

¹Product mixtures, analyzed by GC-MS, contain trace 24-ethyl sterol. In parenthesis is the percent conversion of substrate to product.

²NC, no conversion.