Mechanistic Analysis of a Multiple Product Sterol Methyltransferase Implicated in Ergosterol Biosynthesis in Trypanosoma brucei*§

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Sterol methyltransferase (SMT) plays a key role in sterol biosynthesis in different pathogenic organisms by setting the pattern of the side chain structure of the final product. This catalyst, absent in humans, provides critical pathway-specific enzymatic steps in the production of ergosterol in fungi or phytosterols in plants. The new SMT gene was isolated from Trypanosoma brucei genomic DNA and cloned into an Escherichia coli expression system. The recombinant SMT was purified to homogeneity to give a band at 40.0 kDa upon SDS-PAGE and showed a tetrameric subunit organization by gel chromatography. It has a pH optimum of 7.5, an apparent \( k_{\text{cat}} \) value of \( 0.01 \) s\(^{-1}\), and a \( K_m \) of \( 47 \pm 4 \) \( \mu \)M for zymosterol. The products of the reaction were a mixture of C24-monoalkylated sterols, ergosta-8,24(25)-dienol, ergosta-8,25(27)-dienol, and ergosta-8,24(28)-dienol (fecosterol), and an unusual double C24-alkylated sterol, 24,24-dimethyl ergosta-8,25(27)-dienol, typically found in plants. Inhibitory profile studies with 25-azalanolosterol (\( K_s \) value of 39 nm) or 24(RS),25-epiminolanosterol (\( K_s \) value of 49 nm), ergosterol (\( K_s \) value of 27 \( \mu \)M) and 26,27-dehydrozymosterol (\( K_s \) and \( K_{\text{inact}} \) values of 29 \( \mu \)M and 0.26 min\(^{-1}\), respectively) and data showing zymosterol as the preferred acceptor strongly suggest that the protozoan SMT has an active site topography combining properties of the SMT1 from plants and yeast (37–47% identity). The enzymatic catalysis of this and other SMTs reveals that the catalytic requirements for the C-methyl reaction are remarkably versatile, whereas the inhibition studies provide a powerful approach to rational design of new anti-sleeping sickness chemotherapeutic drugs.

Health in sub-Saharan Africa is threatened by protozoan parasites that generate an estimated 300,000 to 500,000 new cases of African trypanosomiasis, or sleeping sickness, each year. Trypanosoma brucei (TB), the causative agent of sleeping sickness in humans, is a non-photosynthetic flagellate parasite transmitted by the tsetse fly (Glossina). T. brucei has many unusual features, including morphologies at different life stages that contain variant sterol mixtures to respond to the different physiological environments of its human and insect hosts (1). Recent whole-genome sequencing of TB revealed a set of conserved isoprenoid/phytosterol genes that can give rise to squalene and ergosterol (2), consistent with the observation that the parasite synthesizes sterols de novo in either the procyclic or bloodstream form (3, 4).

The sterol methyltransferases (SMT) that catalyze the attachment of a methyl group from AdoMet to the sterol side chain lie on a pathway that is key for the biosynthesis of fungal and plant sterols. These enzymes are additionally unique in that they have no counterpart in humans, they can generate various side chain constructions, and they can serve as the rate-limiting enzymatic step in the overall conversion of lanosterol or cycloartenol to ergosterol and phytosterols involved with membrane structure and function (5). SMT is also of interest enzymologically because the reaction that is catalyzed can be bifunctional with the enzyme accepting different substrate olefins (6). In those protozoans that synthesize ergosterol (7), the C-methylation step makes it possible to develop microbial-specific sterol biosynthesis inhibitors to block a critical slow enzyme in the pathway to treat infected individuals (7–9).

Previous studies on phytosterol synthesis in soluble enzyme preparations from yeast versus vascular plants have demonstrated the presence of two separable activities that catalyze the first and second C1-transfer reactions to produce a C24 methyl and C24 ethyl side chain, respectively (10, 11). However, using cloned enzymes to study product profiles as well as deuterium isotope effects on these enzymatic reactions assayed with native enzymatic preparations has indicated that a single SMT enzyme can produce mixtures of mono-methyl(ene) or di-methyl(ene) sterol products (12–15). SMTs are presently categorized mechanistically based on substrate specificity and product distribution as: 1) SMT1, which catalyzes the first C1-transfer reaction of cycloartenol to 24 (28)-methylene cycloartenol (EC.2.1.1.143, e.g. Glycerine max); 2) SMT1, which catalyzes the first C1-transfer reaction of zymosterol to fecosterol (EC.2.1.1.41, e.g. Saccharomycetes cerevisiae); or 3) SMT2, which catalyzes the second C1-transfer reaction of 24 (28)-methylene loipheno1 and to a mixture of 24 (28)Z-ethylidene lophenol, 24 (28)E-ethylidene lophenol, and 4α-methyl 24β-ethyl cholesta-7, 25 (27)-dienol (EC.2.1.1.143, e.g. Arabidopsis thaliana) (13–16).

A recently cloned SMT from the trypanosomatid parasite Leishmania donovani was shown to support ergosterol production in ERG6 null mutant S. cerevisiae yeasts, suggesting that it falls into the SMT1 category (17). This coupled with the fact that several azasterols were found to inhibit the Leishmania SMT in vitro as well as under physiological conditions (7, 8) suggests that protozoan SMTs generally may share the same mechanism, product diversity, and overall active site topography with fungal SMT1s, thereby paving the way for us to develop anti-parasitic drugs in similar fashion to inhibitors that we have prepared against the fungal SMT (18, 19).

In this study, we have described the characterization of the SMT...
cDNA and recombinant enzyme from TB and reported on the substrate selectivity, inhibitory profile studies, and structure and stereochemistry of the products of the reaction. Quite unexpectedly, we found that the TB SMT generated a set of plant-like products from a zymosterol substrate, including ergosta-8,24(25)-dienol and 24,24-dimethyl ergosta-8,25(27)-dienol, which raises the intriguing possibility that TB has a novel 24-dimethyl sterol pathway that is unlike the fungal ergosterol (C1-ethyl group) or plant sitosterol (C2-ethyl group) pathways and represents a new, catalytically distinct type of sterol methyl transferase that may possess a different active site motif than those of previously described SMTs.

**EXPERIMENTAL PROCEDURES**

**Substrates and Reagents**—The preparation, proof of structure, and purification of 26,27-dehydrozymosterol, 25-azanolasterol, 24(R,S),25-epiminolanosterol, and ergosterol have been described (9, 13–15). Sterol substrates for activity assay were from our sterol collection (13–15). S-Adenosylmethionine (AdoMet) as the iodine salt was purchased from Sigma. [methyl-3H]AdoMet (10–15 Ci/mmol) and [methyl-3H2]AdoMet (99.3% 2H enrichment) were purchased from PerkinElmer Life Sciences and MSD Isotopes, respectively. All other reagents and chemicals were purchased from Sigma or Fisher, unless noted otherwise.

Trypanosoma brucei SMT Cloning, Expression, and Purification—Sequence data for T. brucei SMT were obtained from the web site of The Institute of Genomic Research (www.tigr.org) using Blast search against SMT from S. cerevisiae. The amino acid sequence was aligned with known SMTs (20). The putative TBSMT cDNA was PCR amplified from T. brucei genomic DNA using Vent DNA polymerase (NEB); the details are provided as supplemental information. The PCR product was subcloned into pGEM-T Easy vector (Promega). The correctness of the inserts was confirmed by DNA sequencing. The second codon of the TBSMT cDNA was mutared to alanine (GCT) to optimize foreign gene expression in E. coli (supplemental information); silent mutation (GCC to GCA) was introduced into the third codon to decrease the GC content at the 5′-region. The insert was excised by digestion with NdeI (NEB) and HindIII, cloned into the pCW expression vector, and transformed into the expression host E. coli HMS-174 (Novagen) for ampicillin selection to provide TBSMT-native or TBSMT-His (the details on expression and purification are provided as supplemental information). The protein purity was assessed by SDS-PAGE, and protein concentrations were determined by the Bradford method for total protein (21) or by UV absorption at 280 nm using an extinction coefficient of 49,560 M⁻¹ cm⁻¹ for pure protein. Native molecular weight of the pure protein was determined by gel permeation chromatography (22).

**SMT Assay and Inhibitor Studies**—The standard assay for enzyme activity was conducted in 2-ml vials in final volume of 600 µl in 20 mM phosphate buffer, pH 7.5, 5% glycerol (v/v) with either 50 µM methyl sterol substrate or sterol varialed at 5, 10, 15, 20, 25, 50, and 150 µM and [methyl-3H]AdoMet concentration fixed at saturation of 100 µM (1.2 µCi). The reaction was initiated by addition of 5–15 µg of pure protein or 1–2 mg of total protein and terminated with 500 µl of 10% methanolic KOH. The methylated sterol product was extracted in hexane (Fisher), dried, and the sample analyzed by liquid scintillation counting to determine the conversion rate. The steady-state kinetic parameters, kcat and Km, under initial velocity conditions were calculated by fitting the liquid scintillation data to the Michaelis-Menten equation using the Enzyme Kinetic Module from SPSS Inc. Inhibition of SMT using the native construct was assessed by the graphical procedures provided by the Enzyme Kinetic Module, and rate data were analyzed by linear regression analysis (13). The error in work up of duplicate samples was generally 5% (up to 10% at low conversions). Boiled controls were included in each experiment, and in all cases nonenzymatic formations were negligible. For determination of the pH, temperature, and optimal amount of enzyme, single time point assays containing 2 mg of soluble native SMT were studied as described (13, 23). Variable inhibitor concentrations were performed in the presence of several sterol concentrations, from 5 to 100 µM; fixed substrate was incubated at subsaturating concentrations normally about the Kmw value, and the inhibitor concentration was tested at ranges previously found to inhibit SMT (13, 15).

**Product Analysis**—Radioactivity in organic extracts was determined by liquid scintillation counting (Beckman LS 6500 liquid scintillation counter) of aliquots in a ScintiVerse BD mixture, and all samples were counted to an S.E. <1% at a 3H efficiency of 40%. Structures of enzyme-generated products were determined after reverse phase HPLC purification of the total sterol fraction of the non-saponifiable lipids using either a semi-preparative TSK gel C18-column (Toso Haff) operated at room temperature (eluted with 100% methanol at 2.5 ml/min) or an analytical column operated at 15 °C (eluited with acetonitrile/isopropanol (85:15, v/v) at 1 ml/min). The UV detector was set at 210 nm. In some radioactivity experiments, once the enzyme-generated products were characterized, they were used as authentic carrier standards added prior to solvent concentration to minimize losses and assist the chromatography during HPLC. The details on gas-liquid chromatography-mass spectrometry analysis and NMR spectroscopy are provided in the supplemental information.

**RESULTS**

A TBLASTN search of the T. brucei genome sequence in The Institute of Genomic Research data base using SMT from S. cerevisiae as a query yielded a high probability match to a single 1,077-bp open reading frame from T. brucei, encoding an apparent protein of 359 amino acids. The sequence of the putative TB SMT (Fig. 1) shares 31–47% amino acid identity with other known SMT sequences deposited in the GenBank database and shows strongest homology to SMT1- (59% similarity) rather than SMT2- (45% similarity) type catalysts (Fig. 1). The primary structure of the putative TB SMT contains two highly conserved motifs of 8 to 12 contiguous residues, referred to as Regions 1 and 2 (20). These motifs are arranged in the same order and separated by comparable intervals along the polypeptide side chain of SMTs. Region 1, which stretches from Tyr-81 to Phe-91 (ERG6 numbering system), has been identified by chemical affinity using the yeast SMT1 to be a sterol binding site (24). Region 1 is unique to SMTs and is not found in other AdoMet-dependent methyltransferases. Region 2, which stretches from Lys-124 to Arg-135, has been identified as an AdoMet binding site by site-directed mutagenesis and photoaffinity labeling (21, 26) and is present in many other AdoMet-dependent methyl transferases (25). To determine whether the putative TB SMT was functional to generate the predicted activity, we separately expressed the full-length protein as well as that containing a His6 tag and tested both with zymosterol, a substrate considered to be acceptable to the SMT based on the known sterol composition of protozoa (7).

TB SMT was overexpressed in E. coli HMS-174 cells and yielded target protein migrating on SDS-PAGE with the expected size of ~40 kDa (supplemental Fig. S1 and supplemental Table S1). Fractionation of the total protein starting with the cell lysate generated ~1 or 2 mg of purified His6 tag or native protein. The calculated Ksw value for the native protein using zymosterol as substrate was 47 ± 5 µM, with an apparent kcat value of 0.013 s⁻¹, a pH optimum between 6.8 and 7.5, and temperature opti-
T. brucei Sterol Methyltransferase

| Species          | Type | SMT1 | SMT2 |
|------------------|------|------|------|
| S. cerevisiae    | 1    | MSETELRRKKNFVRGTRLEHGGDIQGTKGLSALMSKNSASQKAYQVYKLKNRDGR---TDRDAEERLEEDYETHS | MSETELRRKKNFVRGTRLEHGGDIQGTKGLSALMSKNSASQKAYQVYKLKNRDGR---TDRDAEERLEEDYETHS |
| G. max           | 1    | MRRKKKSNKNKTYVLC1AGEGSGCLMALALRAMMLNLAMNSAGNTRKWH---CGGS4EGLERMTIM5VYK | MRRKKKSNKNKTYVLC1AGEGSGCLMALALRAMMLNLAMNSAGNTRKWH---CGGS4EGLERMTIM5VYK |
| T. brucei        | 1    | WYS38PLPLKGRKIGLQDLGEEIKDKVIAWAYILQTTYDQY---RKLRRRAEDNY4TGL | WYS38PLPLKGRKIGLQDLGEEIKDKVIAWAYILQTTYDQY---RKLRRRAEDNY4TGL |
| T. cruzi         | 1    | MSGAGPLTILPMMLRRRAEENKIVDTSANFRERR---PEGKADVASYSRKENATTYWYN | MSGAGPLTILPMMLRRRAEENKIVDTSANFRERR---PEGKADVASYSRKENATTYWYN |
| L. major         | 1    | MGCAETTPMLVLRKRDKDEGAIADNFDKPA---FPEKTEDLEERATBY | MGCAETTPMLVLRKRDKDEGAIADNFDKPA---FPEKTEDLEERATBY |
| A. thaliana      | 1    | MSLTLYFTGGLAGAVY1YLCVQPGAAERKDLQGSGSLARRVQQGNKGGNYQYKKSFR---RFIREIAETQVEPFPYD | MSLTLYFTGGLAGAVY1YLCVQPGAAERKDLQGSGSLARRVQQGNKGGNYQYKKSFR---RFIREIAETQVEPFPYD |

The alignment of sterol methyl transferase amino acid sequences (GenBank™ accession numbers) from S. cerevisiae (NP 017206), G. max (T06780), T. brucei (AAZ40214), T. cruzi (EAN81419), L. major (CA09197), and A. thaliana (CA61696), identifies residues conserved in the primary structure are shaded. The sequences were aligned using Align X (Informax Inc.) with default parameters. The deduced substrate preference of SMT that catalyzes the first (\( \Delta^{24}(25)- \) substrate) or second (\( \Delta^{24}(28)- \) substrate) C4-transfer reaction, SMT 1 or SMT2, is reported. Sterol and AdoMet binding sites are indicated as Region I and Region II, respectively.

| Species          | Type | SMT1 | SMT2 |
|------------------|------|------|------|
| S. cerevisiae    | 1    | DENVFRKIKVAYKIPKDDGSLMDQVYKSPORTN---DDDRDDTPTFYFGTVYKLQIATIGTVY | DENVFRKIKVAYKIPKDDGSLMDQVYKSPORTN---DDDRDDTPTFYFGTVYKLQIATIGTVY |
| G. max           | 1    | PQVEFSFKEPALFAGLGLIGGEDKRTCKLEALQAEGVETQGTVL---VYMDPSTLQKDYSGILYK | PQVEFSFKEPALFAGLGLIGGEDKRTCKLEALQAEGVETQGTVL---VYMDPSTLQKDYSGILYK |
| T. brucei        | 1    | PDIENQKYVTSSQMLQYEDQSLWADLQKEDDVERKAQTKLAG---FEEPDDDPGLYK | PDIENQKYVTSSQMLQYEDQSLWADLQKEDDVERKAQTKLAG---FEEPDDDPGLYK |
| T. cruzi         | 1    | PDIENQKYVTSSQMLQYEDQSLWADLQKEDDVERKAQTKLAG---FEEPDDDPGLYK | PDIENQKYVTSSQMLQYEDQSLWADLQKEDDVERKAQTKLAG---FEEPDDDPGLYK |
| L. major         | 1    | PVEFSFKEPALFAGLGLIGGEDKRTCKLEALQAEGVETQGTVL---VYMDPSTLQKDYSGILYK | PVEFSFKEPALFAGLGLIGGEDKRTCKLEALQAEGVETQGTVL---VYMDPSTLQKDYSGILYK |
| A. thaliana      | 1    | ADVMDTVGLYKQDIAATG---NFTRWIRLGSQ | ADVMDTVGLYKQDIAATG---NFTRWIRLGSQ |

The 40-kDa SMT eluted from the SDS-PAGE gel revealed the N-terminal set of amino acids MSAGS (Texas Tech University Core Facility), consistent with the predicted amino acids in the primary structure (Fig. 1). The protein was analyzed on gel permeation chromatography and found to possess a native molecular mass of ~160 kDa, suggesting the expected tetramer subunit organization. Initial assay with zymosterol and analysis of the enzyme-generated product by gas chromatography-mass spectrometry revealed the SMT catalyzed the formation of four products formed in an approximate 62:13:13:12 ratio (Fig. 2). For bio-synthetic reasons, the C4-methyl product ergosta-8,24 (28)-diol (fecosterol; \( I \)) was expected; however, three other 24-methyl sterols were detected corresponding to ergosta-8,25 (27)-diol (2), ergosta-8,24 (25)-diol (4), and 24,24-dimethyl-ergosta-8,25 (27)-diol (5). The 500-MHz \(^1\)H NMR spectra of the HPLC pure ergostenols generated by the TB SMT (Table 2) confirmed the structures assigned in the chromatogram reported in Fig. 2. Product stereochemistry at C24 and regiochemistry of the double bond in the sterol side chain of each compound was determined by comparison of the \(^1\)H NMR signals at C21 and C28, and those signals associated with the olefinic bonds at \( \Delta^{24}(28) \) versus \( \Delta^{24}(27) \) to reference specimens (14, 20). The \(^1\)H NMR chemical shifts of the pure compound 5 unambiguously reveal the geminal dimethyl group at C24 (signals resonating at 1.007 and 1.012 ppm) and a double bond at C25 (27) (signals at 4.654 and 4.717 ppm). The side chain structures of compounds 4 and 5 have been reported in plants (27, 28). Taken together, these spectral data prove a doubly alkylated side chain at carbon-24, and contrary to expectation, the 2 “extra methyls” are not in the normal 24-ethyl arrangement but in the unusual 24,24-dimethyl arrangement.

Deuterium-labeled methyl AdoMet (\([\text{methyl}-^2\text{H}_3]\)AdoMet) was employed to assess the course of the enzymatic reaction and to confirm that all the products were generated by TB SMT. \([\text{methyl}-^2\text{H}_3]\)AdoMet was incubated with TB SMT in the usual way and resulting enzyme-generated products analyzed by gas chromatography-mass spectrometry. When compared with the mass spectra of control specimens (Table 3), all four of the enzyme-generated methyl products were labeled with deuterium atoms (Table 3). Ergosta-8,25 (27)-diol 2 and ergosta-8,24 (25)-diol 4 incorporated three deuterium atoms into the side chain.
**T. brucei Sterol Methyltransferase**

In an effort to establish the potency of possible ergosterol biosynthesis inhibitors against the TB SMT, the substrate analogs 25-azalanossterol 16, 24(R,S)-25-epiminolanosterol 17, ergosterol 18, and 26,27-dehydrozymosterol 19 were assayed with the protozoan enzyme (Fig. 4). The four compounds were chosen based on their demonstrated performance to inhibit the SMT-catalyzed reaction in the direction of C24-methyl(ene) product and their different mechanisms to impair SMT action. Azasterols and related “charged” inhibitors such as 25-azalanossterol and 24(R,S)-25-epiminolanosterol have been used as probes of sterol biosynthesis. They will bind reversibly to SMT and exhibit non-competitive type kinetics against zymosterol (5, 20). They are believed to be “high energy intermediate analogs” in which the protonated, ammonium form of the molecule (presumably generated at physiological pH) mimics the putative carbocationic intermediate in the C-methyl transfer reaction (11). In contrast to lanosterol as a substrate, which in some cases fails to bind productively to an SMT, the 24-carbon ammonium-containing lanosterols bind tightly to SMTs for mechanistic reasons and block catalysis. When these inhibitors are added in vivo (M’ enhanced by three mass units from M’ 398 to 401 atomic mass units), ergosta-8,24 (28)-diol 3 incorporated two deuterium atoms (M’ enhanced by two mass units from M’ 398 to 400 atomic mass units), and 24,24-dimethyl ergosta-8,25 (27)-diol incorporated six deuterium atoms into the side chain (M’ enhanced by six mass units from 412 to 418 atomic mass units). The observation that ergosta-8,24 (25)-diol carried three deuterium atoms and 24,25-dimethyl ergosta-8,25 (27)-diol carried six deuterium atoms is noteworthy. Thus far, for mechanistic and biosynthetic reasons (29, 30), the formation of the ergosta-8,24 (25)-diol side chain is thought to result from the isomerization of the 24 (28)-side chain, which if this were the case then 4 should carry two rather than three deuterium atoms following assay with [methyl-2H3]AdoMet. In addition, if sterol 5 were a 24-ethyl sterol such as a stigmasterol-type compound (7) rather than a 24,24-dimethyl sterol, the deuterium-labeled compound following assay with [methyl-2H3]AdoMet will carry four deuterium atoms (14). These results suggest that the SMT reaction proceeds stepwise, undergoing first a C1-transfer reaction to give three products, ergosta-8,25 (27)-diol, ergosta-8,24 (28)-diol, and ergosta-8,24 (25)-diol followed by a second C1-transfer reaction to give 24,24-dimethyl ergosta-8,25 (27)-diol. To determine the substrate specificity for the second C1-transfer reaction, 100 μM [methyl-2H3]AdoMet and 100 μM each of the three monoalkylated sterols generated by the TB SMT during the first C1-transfer reaction were assayed separately with 1 ml of soluble enzyme (5 mg/ml) at 37°C for 8 h. Only ergosta-8,24 (25)-diol was converted to product by the TB SMT with 95% conversion of substrate to 24,24-dimethyl ergosta-8,25 (27)-diol under the assay conditions.

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### TABLE I

**Kinetic parameters of sterol substrates assayed with T. brucei sterol methyl transferase**

| Substrate | Structure | V | V/K (catalytic competence) |
|-----------|-----------|---|----------------------------|
|           |           | μmol/min/mg |                           |
| Lanosterol| 6         | NA | 0 (0)                      |
| 31-Norlanosterol | 7 | 35 | 1.5 (8)                    |
| 14r-Methyl cholesta-8, 24-dienol | 8 | 46 | 3.1 (16)                   |
| 4r-Methyl cholesta-8, 24-dienol | 9 | 28 | 4.0 (21)                   |
| Cholesta-8, 24-dienol | 1 | 47 | 19.2 (100)                 |
| Cholesta-7, 24-dienol | 10 | 40 | 15.5 (81)                  |
| Cholesta-5, 24-dienol | 11 | 52 | 5.0 (26)                   |
| 24,25-Dehydro-pollinastanol | 12 | 30 | 2.7 (14)                   |
| 31-Norcylosterol | 13 | 28 | 1.0 (5)                    |
| Cycloartenol | 14 | NA | 0 (0)                      |
| 24(28)-Methylene lophenol | 15 | NA | 0 (0)                      |

*See Fig. 3 for a key to the structures.

% Transformation of substrate compared to zymosterol 1 activity (in bold).
to plants, fungi, or protozoa, Δ24-sterols accumulate, ergosterol homeostasis is disrupted, and cell growth is impaired (5, 19, 20). In assays with the TB SMT, 25-azalanosterol and 24(28)-methylene lophenol or the type of reaction mechanism (non-stop versus stepwise) appear to be quite dissimilar (though all "concerted" mechanisms generate and stabilize high energy intermediates). The low mass end fragmentation pattern of sterols assayed with AdoMet or in brackets [methyl-H]AdoMet and zymosterol.

**TABLE II**

H-NMR assignments of T. brucei enzyme-generated products

The samples, purified by HPLC, were dissolved in CDCl₃ and the values reported in ppm relative to Me₄Si. s, singlet; d, doublet; m, multiplet.

| Signal | 2     | 3     | 4     | 5     |
|--------|-------|-------|-------|-------|
| H₁⁻¹₈ | 0.948 | 0.952 | 0.951 | 0.597 |
| H₂⁻⁹⁻ | 0.603 | 0.614 | 0.612 | 0.947 |
| OH⁻３⁻ | 3.615 | 3.616 | 3.621 | 3.608 |
| H₂⁻⁶⁻ | 1.636 | 1.023 | 1.635 | 1.633 |
| H₂⁻⁷⁻ | 4.657 | 1.028 | 1.617 | 1.681 |
| H₁⁻₂₈⁻ | 0.986 | 1.629 | 1.007 |
| H₁⁻₂₉⁻ | 1.012 |

**TABLE III**

Mass spectra of T. brucei enzyme-generated sterols following activity assay with AdoMet or [methyl-2H₃]AdoMet and zymosterol

High mass end fragmentation pattern of sterols assayed with AdoMet or in brackets [methyl-H]AdoMet.

**DISCUSSION**

SMT from *T. brucei*, like many other SMTs of plant and fungal origin, is capable of producing multiple products, probably as a consequence of the similar ionic C-methylation pathways employed for catalysis. The binding and catalysis of substrates to these SMT enzymes are considered to be determined by active site polar and non-polar steric interactions as envisaged in the "steric-electric plug" model of the SMT (5). However, in the absence of a crystal structure for this class of AdoMet-dependent methyltransferase, defined interactions have yet to be demonstrated to control specificity. However, the enzyme-generated product profiles are very different for TB and yeast SMTs. The yeast SMT catalyzes zymosterol to a single product, whereas the TB SMT catalyzes zymosterol to multiple products. Plant SMTs can also bind and catalyze zymosterol efficiently, such as the SMT2 from *A. thaliana* (46% similarity with TB SMT), and produce the same Δ24(28)-methylene product as the yeast SMT1 (31). Information from sequence alignment revealed the derived amino acid sequence of *T. cruzi* and *Leishmania major* have significant similarity to TB SMT and suggests that all these protozoan SMTs are of the SMT1 type. However, it is less obvious how the protozoan SMT1, yeast SMT1, plant SMT1, or plant SMT2 are related, as either the optimal substrates (zymosterol versus cycloartenol and 24(28)-methylene lophenol) or the type of reaction mechanism (non-stop “concerted” versus stepwise) appear to be quite dissimilar (though all these enzymes likely generate and stabilize high energy intermediates). Our results also showed that the four inhibitors tested with the SMT manifest distinct modes of inhibition in analogous manner to that observed for other SMTs. As expected, high homology of the SMTs studied thus far, the sequence and the structure of the protozoan SMT are expected to closely resemble that of the plant and fungal SMTs. However, the different inhibition potencies of these compounds by a factor of five against the TB SMT compared with the yeast SMT (both SMTs recognize zymosterol as the optimal substrate) (5, 6, 9) clearly indicate...
the existence of subtle differences between these two enzymes, perhaps because of sequence variations at or near the active site.

Studies on SMT mechanisms have shown that the C-methylation reaction can be considered in its initial step as a nucleophilic attack by the $\Delta^{24}$-double bond of acceptor sterols on the sulfonium methyl group of AdoMet, followed by formation of one or more C24-methyl(ene) products (5). For mechanistic reasons, the SMT is limited to a select number of products that can be generated from a $\Delta^{24}$ (25)-sterol substrate (29, 30). Two alternatives for deprotonation of the high energy intermediates A and B (Fig. 5) yield $\Delta^{25}$ or $\Delta^{24}$-olefins A-1 and B-1, respectively. Arigoni (30) postulated that the methyl addition step and 1,2-hydride shift of H24 to C25 during SMT catalysis is common to the formation of both ergosta-8,24 (28)-dienol and ergosta-8,25 (27)-dienol and that the deprotonation step is similar (i.e. the proton is removed from the methyl group that is aligned on the same side of the side chain as the hydrogen atom of the isopropyl cationic center of the high energy intermediate A). Because in all other previously studied SMTs the enzymes failed to generate ergosta-24 (25)-dienol as a methylation product, it seemed reasonable that the SMT lacked an active site base to abstract the H24 as it migrates between C24 and C25 in the formation of ergosta-8,24 (28)-dienol (31). In this study we discovered that the TB SMT can produce a mixture of four C24-methyl sterols, one of which contains the 24-methyl $\Delta^{24}$ (25)-double bond-containing side chain. The incorporation of three rather than two deuterium atoms at C24 in ergosta-8,24 (25)-dienol from assay with [methyl-$^\text{H}_2$]AdoMet and the structures and stereochemical assignments of the enzyme-generated products indicate the methylation pathway can proceed stepwise via cations A and B (path a) to produce multiple products (Fig. 5). However, it remains enigmatic how the active site residues involved with molecular recognition and catalysis precisely control the resulting reaction progress involving methylation, hydrbide shift, and deprotonation and ultimately quench the positive charge of the high energy intermediates A and B. Two possibilities can be imagined. First, through a conformational shift, a mobile, contact amino acid that also doubles as a deprotonating base could abstract protons sequentially from C27 and C28 and then capture H25 as it slides back to C24 to eliminate the positive charge of cation B. This implies the sterol side chain assumes a preferred conformation at binding, the topography of the active center changes during catalysis, and a single active site base is positioned to mediate multiple deprotonations. A second possibility involves a flexible sterol side chain that moves, relative to a set of fixed enzyme nucleophiles, which requires alternately oriented binding modes for the $\Delta^{24}$-acceptor. The product diversity involving the deprotonation of the first C1-transfer activity leading to the formation of ergosta-8,24 (25)-dienol suggests that a proton in this structure is chemically and geometrically distinct from that lost in the formation of ergosta-8,24 (28)-dienol and ergosta-8,25 (27)-dienol, consistent with multiple amino acid residues acting as active site bases in the TB SMT. In this connection, it is interesting that the different spatial arrangements of the sterol side chain in the activated complex may hold the key to the generation of taxa-specific C1-transfer reaction pathways. In our stereochemical model for substrate recognition and catalysis (5), we assume that differences in product specificity among the SMT enzymes have arisen through mutations that change the shape of the C1-transfer reaction active sites and/or the positions of the crucial functional groups.

Natural product chemists recognize the size and direction of the 24-alkyl group in the sterol side chain to represent whether the organism is operating a primitive or advanced sterol biosynthetic pathway (a 24B-methyl group synthesized in primitive organisms and a 24a-ethyl group synthesized in advanced organisms) (32). The unusual product composition generated by the TB SMT (i.e. 24B methyl $\Delta^{25}$-olefins that predominate in the sterol mixture) suggests that this enzyme is more primitive than other SMTs studied to date. The operation of either a cycloartenol- or lanosterol-based pathway leading to $\Delta^{25}$-sterols is another phylogenetic marker in sterol biosynthesis and indicates whether the organism evolved from a photosynthetic or non-photosynthetic lineage (32, 33). Plant-like traits have been identified with substrate specificity or product diversity in the sterol pathway of T. brucei (this study and Ref. 34), and they have been reported in other aspects of the biology of Trypanosoma parasites (35) for which an algal origin has been hypothesized (35). However, we surmise that TB evolution avoided an ancestral plastid endosymbiont precursor, as others have also proposed (2), because lanosterol is formed exclusively by the cloned TB synthase (36). The summation of these results reveals an unusual trypanosome sterol biosynthetic pathway suggesting that further understanding of catalytic control in the C-methylation reaction may contribute to a complete unraveling of sterol phylogenies in TB and to the rational design of mechanism-based inhibitors that bind specifically to SMT and impair only the C-methylation step in sterol biosynthesis. This stereochemical evaluation of an SMT from protozoa together with our earlier studies of this class of catalyst (5) represents the first such examination of the origin and regulation of a new reaction pathway in which absolute mechanistic inferences regarding primary structure, substrate specificity, and product outcomes are not possible.

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