

Lanosterol Biosynthesis in the Prokaryote *Methylococcus Capsulatus*: Insight into the Evolution of Sterol Biosynthesis

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A putative operon containing homologues of essential eukaryotic sterol biosynthetic enzymes, squalene monooxygenase and oxidosqualene cyclase, has been identified in the genome of the prokaryote *Methylococcus capsulatus*. Expression of the squalene monooxygenase yielded a protein associated with the membrane fraction, while expression of oxidosqualene cyclase yielded a soluble protein, contrasting with the eukaryotic enzyme forms. Activity studies with purified squalene monooxygenase revealed a catalytic activity in epoxidation of 0.35 nmol oxidosqualene produced/min/nmol squalene monooxygenase, while oxidosqualene cyclase catalytic activity revealed cyclization of oxidosqualene to lanosterol with 0.6 nmol lanosterol produced/min/nmol oxidosqualene cyclase and no other products observed. The presence of prokaryotic sterol biosynthesis is still regarded as rare, and these are the first representatives of such prokaryotic enzymes to be studied, providing new insight into the evolution of sterol biosynthesis in general.

Introduction

Sterol biosynthesis is generally associated with eukaryotic kingdoms of life, where the end-product molecular structure differs, including cholesterol found in most animals, ergosterol in yeasts and fungi, and phytosterols in plants (for review, Bloch 1992; Nes 1994), but the origin of sterol biosynthesis is an active subject of investigation and debate. The initial steps of sterol biosynthesis are conserved in all eukaryotes and emanate from acetyl CoA, involving the biosynthesis of isopentenyl pyrophosphate and the subsequent condensation of 6 such molecules to form squalene. The first parental sterol molecule results from the cyclization of oxidosqualene, and this molecule is then further tailored by up to 20 additional steps required for the biosynthesis of the final sterol product, e.g., cholesterol. During the last 40 years, much scientific scrutiny and experimental investigation has focused on 3 postsqualene steps of this pathway: epoxidation of the hydrocarbon squalene catalyzed by squalene monooxygenase (SM), cyclization of squalene epoxide to form the initial sterol molecule (lanosterol or cycloartenol) catalyzed by oxidosqualene cyclase (OC); and finally oxidative removal of C32 of lanosterol/cycloartenol catalyzed by cytochrome P450 14-demethylase (CYP51) (fig. 1).

The pathways that have evolved also show biosynthetic molecular diversity, with either a lanosterol intermediate being observed, as in fungi and animals, or else a cycloartenol route being observed, as in plants (Corey, Matsuda, and Bartel 1993). The routes are of considerable interest in considerations on the origin of sterol biosynthesis and whether one or the other route is the most ancient in this basic biosynthetic pathway in the cell that requires molecular oxygen. The presence of a lanosterol pathway in *Methylococcus capsulatus* contributes to these discussions and

is the subject of our recent work after identifying the first proven prokaryotic sterol biosynthetic gene/protein (Jackson et al. 2002). The importance of sterols in eukaryotes is well established because they modulate membrane fluidity and also serve as precursor molecules for hormone and brassinosteroid biosynthesis. In contrast, putative roles of sterol in bacteria are relatively rare in nature and poorly understood. Very few bacteria have been shown conclusively to synthesize sterol *de novo*, and genomic bioinformatic analysis confirms this. Although homologues of sterol biosynthetic genes are sometimes observed within sequenced prokaryotic genomes, this may represent recruitment to new cellular functions (Kelly et al. 2003; Volkman 2003). Recently, phylogenetic and biochemical analyses of the bacterium *Gemmata obscuriglobus* revealed the presence of sterol and of putative genes encoding the sterol biosynthetic enzymes squalene monooxygenase and oxidosqualene cyclase (Pearson, Budin, and Brocks 2004), although the cytochrome P450 sterol demethylase step is absent in this organism. This also followed the identification of a cycloartenol synthase and sterols in *Stigmatella aurantiaca* (Bode et al. 2003). However, of historical importance, the first bacterium shown to conclusively make sterol was *Methylococcus capsulatus*, a methylophilic which grows on methane and has been shown to contain the sterols 4 α -methyl-5 α -cholest-8(14)-en-3 β -ol, 4,4-dimethyl-5 α -cholest-8(14)-en-3 β -ol, 4 α -methyl-5 α -cholest-8(14)-24-dien-3 β -ol, and 4,4-dimethyl-5 α -cholest-8(14)-24-dien-3 β -ol (Bird et al. 1971; Bouvier et al. 1976). Presumably, the presence of sterol within this organism offers a selective advantage to adapt to conditions within the environment it inhabits. Specifically, it is assumed sterols provide an advantage for membrane architecture and robustness, although other roles cannot be excluded. Identification of such sterols in *M. capsulatus* indicates that genes encoding squalene monooxygenase, oxidosqualene cyclase, and lanosterol 14 α -demethylase must be present. Recently, we have isolated and characterized the *M. capsulatus* lanosterol 14 α -demethylase (MCCYP51FX) (other names CYP51, P450_{14DM}) (Jackson et al. 2002). This protein was, surprisingly, a soluble form, unlike the membrane-bound eukaryotic counterparts (Lamb et al. 1999), and represented a new class of cytochrome P450 (CYP) being fused at the C-terminus to a ferredoxin domain.

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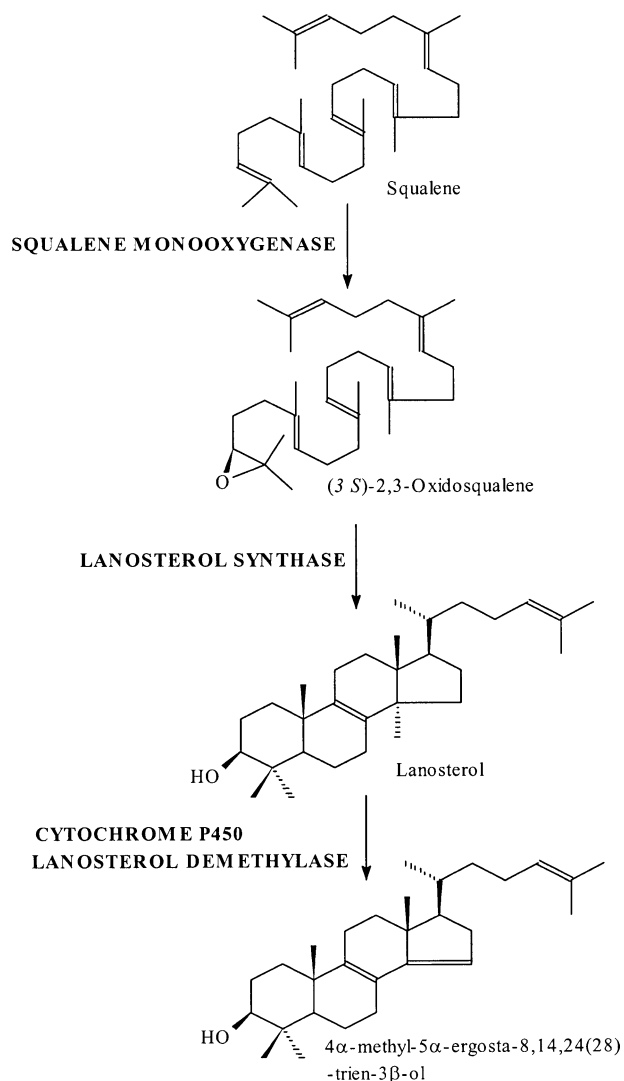


FIG. 1.—The postsqualene biosynthetic reactions of squalene monooxygenase, lanosterol synthase, and lanosterol demethylase involved in the conversion of squalene to 4-methyl-5-ergosta-8,14,24(28)-trien-3-ol.

In the present paper we describe the complete molecular characterization of the initial 3 postsqualene steps of sterol biosynthesis in *M. capsulatus*. Cloning, expression, and purification of each enzyme was carried out and activity established. We provide evidence of a protein scaffold for these enzymes of sterol biosynthesis whereby a complex between the soluble oxidosqualene cyclase and sterol demethylase enzymes forms with the membrane-bound squalene monooxygenase. This is in direct contrast to the eukaryotic enzymes, where all are membrane bound in the endoplasmic reticulum. The implication of our results regarding the evolution of sterol biosynthesis, following the introduction of oxygen into the early atmosphere, are discussed.

Materials and Methods

Genome Analysis

Sequence data for the genome of *M. capsulatus* was obtained from The Institute for Genomic Research website

at <http://www.tigr.org>. A BLASTP (Altschul et al. 1990) homology search of the translated *M. capsulatus* genome was made using the protein sequence of the *Saccharomyces cerevisiae* squalene monooxygenase (Erg1p) and lanosterol synthase (oxidosqualene cyclase) (Erg7p) (Corey, Matsuda, and Bartel 1994; Shi, Buntel, and Griffin 1994). Putative *M. capsulatus*-translated squalene monooxygenase (TIGR Accession YP_115265; Locus tag MCA2872; Gene id: monooxygenase family protein) and oxidosqualene cyclase (TIGR Accession YP_115266; Locus tag MCS2837; Gene id: squalene cyclase family protein) gene products were BLAST searched against Swiss-Prot protein sequence database to identify putative functional homologues. All data were aligned as protein sequences in the appropriate protein family. Multiple sequence alignments were carried out using CLUSTALW (Thompson, Higgins, and Gibson 1994). Alignments were verified by the checking of known conserved motifs in each family alignment. Unrooted phylogenetic trees were created by using TREEVIEW (Page 1996).

Bacterial Strains, Media, and Growth Conditions

The *M. capsulatus* type strain ACM3302 (BATH) was obtained from the National Collection of Industrial and Marine Bacteria (NCIMB Ltd., Aberdeen, Scotland). Cells were cultured in 250 ml minimal salts medium (Satoh et al. 1993) at 37°C in an atmosphere of methane/air, 1:1 v/v. After 3 days incubation, cells were harvested by centrifugation and washed once in 10 ml of 0.1 mM Tris-HCl, pH 7.5.

DNA Extraction and PCR Amplification of *M. capsulatus* Squalene Monooxygenase and Oxidosqualene Cyclase

Fifty milligrams of cells were resuspended in 500 µl of sterile, double-distilled water and boiled for 10 min. The cell debris was pelleted by centrifugation at 10,000 × g for 5 min, and the supernatant aspirated and snap-cooled on ice. Primers were designed to amplify the *M. capsulatus* squalene monooxygenase open reading frame (ORF):

Forward: 5' CGC CAT ATG AGT TCG ATT GAA TTT G

Reverse: 5' GCT AAG CTT TCA GTG ATG GTG ATG TTT CGC CCG CAG CGC CTG and the oxidosqualene cyclase ORF

Forward: 5' AGC TCA TAT GGC AGG CGG GGT TGC ACG

Reverse: 5' GCA TAA GCT TCA ATG GTG ATG GTG TCT GCG ATA ACC CGT GTC

The primers incorporate unique *Nde* I (underlined) and *Hind* III (double underlined) cloning sites and a C-terminal polyhistidine tag (bold) for downstream purification of the expressed protein. For optimal oxidosqualene cyclase expression, the second codon was altered to encode alanine (GCG) rather than proline (CCC). Each PCR reaction was assembled using 10 µl of 10× PCR buffer, 6 µl of magnesium chloride (25 mM); 1.5 µl of dNTPs (25 mM each of dATP, dGTP, dCTP, dTTP); 1.5 µl each primer (10 pmol), 62 µl PCR-grade water; 6 µl dimethyl sulfoxide (Sigma), 10 units of *Taq* DNA polymerase, and 1 µl of diluted DNA

template (all PCR reagents and restriction enzymes supplied by Promega). Amplification was carried out by denaturation at 95°C for 120 s, then 35 cycles of denaturation at 95°C for 45 s; annealing at 50°C for 45 s, and extension at 72°C for 120 s. A terminal extension of 10 min at 72°C completed the reaction.

Cloning and Expression of *M. capsulatus* Squalene Monooxygenase and Oxidosqualene Cyclase

The PCR products were purified from an agarose gel and cloned into the pGEM-T Easy vector (Promega). Each insert was excised, and double-digestion with *Nde* I and *Hind* III restriction enzymes (Promega) was performed. The product was cloned into the pET17-b expression vector (Novagen) and transformed into *E. coli* BL21-pLYS cells (LifeTech). For protein expression, transformed cells were grown in Terrific Broth (tryptone, 12 g/l; yeast extract, 24 g/l; glycerol, 4 ml/l; KH₂PO₄, 2.3 g/l; K₂HPO₄, 12.5 g/l) for 5 h at 37°C with 100 µg/ml ampicillin. Expression was induced by addition of isopropyl α -D-thiogalactopyranoside (IPTG) to a final concentration of 1 mM, and incubation continued at 24°C for 20 h.

Purification of *M. capsulatus* Squalene Monooxygenase, Oxidosqualene Cyclase, and Sterol Demethylase/Ferredoxin Fusion

Eight liters of *E. coli* expressing the desired protein was pelleted by centrifugation at 1,500 \times g and resuspended in 200 ml potassium phosphate buffer, pH 7.4. Cells were broken following 2 passages through a C5 homogeniser (Avestin) using an operating pressure of 15,000 psi. The lysed cells were centrifuged at 10,000 \times g to remove unbroken cells and cell debris. The cytosolic fraction was separated from the membrane fraction by ultracentrifugation at 100,000 \times g for 45 min. Purification of squalene monooxygenase required prior solubilisation from the membrane, which was achieved by treating membranes with 1% (w/v) sodium cholate and stirring for 1 h at 4°C followed by ultracentrifugation at 100,000 \times g for 45 min. Each protein was isolated using a Ni²⁺-nitrilotriacetic acid (NTA) affinity column (Qiagen) equilibrated with 50 mM potassium phosphate/20% (v/v) glycerol pH 7.4 (buffer A). The column was washed with buffer A containing 50 mM glycine and 0.5 M sodium chloride, and recombinant protein was eluted with buffer A containing 100 mM imidazole. Purified proteins were dialyzed overnight against 50 mM Tris-HCl, pH 7.4 containing 10% (v/v) glycerol.

Reconstituted Enzymatic Activity

Squalene monooxygenase activity was measured as described previously (Sato et al. 1993). The standard assay mixture contained squalene monooxygenase, spinach ferredoxin, and spinach ferredoxin reductase in a ratio 1:10:3. Following dispersal of 30 nmol squalene in 50 nmol dilaurylphosphatidylcholine, the enzyme mixture was added, and

NAPDH (1 mM final concentration) was added to initiate the reaction. Reactions were incubated for 30 min before termination by the addition of 3 ml of methanol, and samples were extracted as described below. Oxidosqualene cyclase activity was determined using 1 nmol purified enzyme and 30 nmol oxidosqualene (Echelon Biosciences Inc., Salt Lake City, UT). Assay of catalytic activity was carried as described previously (Dean et al. 1967; Moore and Schatzman 1992). Briefly, oxidosqualene in chloroform was added to a 1% (v/v) solution of Tween 80 in methanol-water (1:4, v/v), and the organic solvent was evaporated in a stream of nitrogen. To the residual aqueous suspension of substrate, phosphate buffer at 40°C was added, and the mixture was agitated, resulting in the formation of an emulsion. Oxidosqualene cyclase was then added to the mixture, the reaction volume was adjusted to 1 ml, and samples were incubated at 37°C for 15 min. Control experiments included absence of enzyme and boiled protein. Reactions were stopped by the addition of 3 ml methanol, and sterol substrate and product were extracted by incubation with 90% (w/v) ethanolic potassium hydroxide at 80°C for 1 h in a preheated water bath. Following silylation for 1 h at 60°C with 50 µl BSTFA (*N,O*-bis[Trimethylsilyl]trifluoroacetamide) in 50 µl toluene, sterol substrates and metabolites were clearly separated and identified by GC/MS (VG 12-250 [VG BIOTECH]). A value for turnover (nmol lanosterol produced/min) was determined using the amount of substrate added, the conversion ratio, and extraction efficiency.

General Methods

Reduced carbon monoxide difference spectra for quantification of cytochrome P450 content were measured and calculated according to the method described by Omura and Sato (1964). Protein quantification was performed by using the Bicinchoninic acid assay (Sigma). Unless otherwise stated, all chemicals were supplied by Sigma Chemical Company (Poole, Dorset, UK).

Results

Discovery of Squalene Monooxygenase and Oxidosqualene Cyclase by Bioinformatic Analysis

Using *S. cerevisiae* Erg1p and Erg7p as BLASTP queries against the translated *M. capsulatus* genome, predicted gene products with homology to squalene monooxygenases and lanosterol synthases, respectively, were detected from a range of diverse organisms. The putative *M. capsulatus* squalene monooxygenase (TIGR Accession YP_115265; Locus tag MCA2872; Gene id: monooxygenase family protein) was predicted to be 445 amino acids in length and showed strongest homology (46% (219/445) identical residues; 59% (289/445) identical or similar residues) to the recently described putative squalene monooxygenase from *G. obscuriglobus* (Pearson, Budin, and Brocks 2004). The putative *M. capsulatus* oxidosqualene cyclase (TIGR Accession YP_115266; Locus tag MCS2837; Gene id: squalene cyclase family protein) was predicted to be 670 amino acids in length. *M. capsulatus* oxidosqualene cyclase showed strongest homology

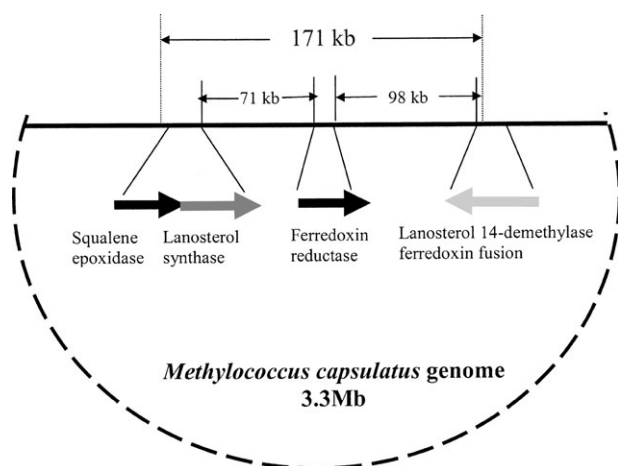


FIG. 2.—Schematic diagram showing the map position of the post squalene sterol biosynthetic genes on the *M. capsulatus* chromosome. Locations of the genes encoding squalene monoxygenase, lanosterol synthase and lanosterol 14 α -demethylase were determined by analysis of the recently sequenced chromosome (<http://www.tigr.org>). Position of a ferredoxin reductase, which can interact with and drive lanosterol 14 α -demethylase and which may also provide the necessary reducing equivalents for squalene monoxygenase function, is also included.

(values identical to that of squalene monoxygenase above) to the putative cyclase from *G. obscuriglobus* (Pearson, Budin, and Brocks 2004; Summons et al. 2006), (fig. 2). *M. capsulatus* oxidosqualene cyclase studied here also showed significant homology to a recently described cycloartenol cyclase from the myxobacterium *S. aurantiaca* (Bode et al. 2003). The closest eukaryotic homologue was lanosterol synthase from *Rattus norvegicus* (Abe and Prestwich 1995), with 38% (172/449) identical residues (fig. 2). Unlike *M. capsulatus*, *G. obscuriglobus* was shown to biosynthesize parkeol and low levels of lanosterol as the final sterol products, necessitating a requirement for only squalene monoxygenase and oxidosqualene cyclase (Pearson, Budin, and Brocks 2004; our personal observations). In contrast, *M. capsulatus* possesses a third post-squalene sterol biosynthetic gene, lanosterol 14 α -demethylase (TIGR Accession YP_115112; Locus tag MCA2711; Gene id: CYP 51), resulting in 14 α -demethylated sterols being observed within this prokaryote. As such, our previous report of a sterol 14 α -demethylase from *M. capsulatus* (MCCYP51FX; Jackson et al. 2002) was the first prokaryotic enzyme of sterol biosynthesis to be studied where a firm identification of sterols had already been established (Bird et al. 1971; Bouvier et al. 1976).

Cloning, Expression, and Purification of Squalene Monoxygenase and Oxidosqualene Cyclase

M. capsulatus squalene monoxygenase and oxidosqualene cyclase 1350 and 2196 bp open reading frames were amplified by PCR, and the sequences of each clone used subsequently was shown to be identical to that revealed from the genome sequencing project. Both squalene monoxygenase and oxidosqualene cyclase were produced as recombinant proteins in *E. coli* following heterologous

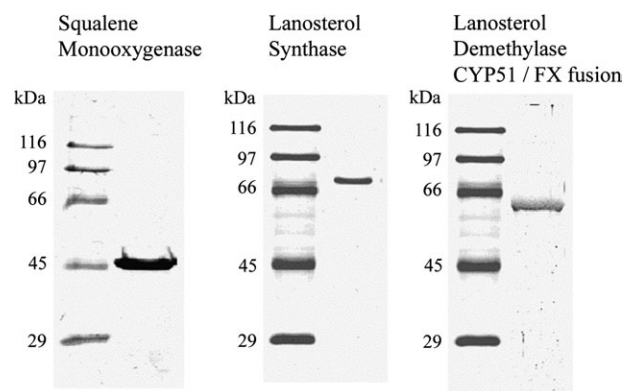


FIG. 3.—Protein purification analysis showing the isolation of *M. capsulatus* squalene monoxygenase (A), lanosterol synthase (B), and CYP51FX (C) as monitored by Coomassie Brilliant Blue staining. Each protein was purified via 4 \times His tag purification technology using Ni²⁺-NTA affinity chromatography. Lane 1, molecular weight standards; lane 2, purified recombinant protein following 2 passes over Ni²⁺-NTA.

expression from pET17b and after a C-terminal 4 \times histidine tag was added. In agreement with a predicted membrane anchor on analysis of the amino acid sequence of the squalene monoxygenase, cell fractionation following expression revealed the recombinant protein to be membrane bound. After solubilization from the membrane and 2 consecutive Ni²⁺-NTA-affinity chromatography purification steps, SDS-PAGE showed enrichment of a major protein at approximately 45 kDa (fig. 3), being comparable with the predicted molecular weight of the squalene monoxygenase gene product. In contrast, cell fractionation revealed oxidosqualene cyclase to be soluble, in a similar fashion to the sterol 14-demethylase, being located predominantly in the cytosolic fraction following ultracentrifugation at 100,000 \times g. For successful expression in *E. coli* it was optimal that the second codon was altered to encode alanine (GCG) rather than proline (CCC), similar to previous observations on successful recombinant protein expression in *E. coli*. Purification using Ni²⁺-NTA-affinity chromatography followed by SDS-PAGE showed enrichment of a major protein at approximately 72 kDa (fig. 3) being comparable with the predicted molecular weight of the oxidosqualene cyclase gene product (76 kDa).

M. capsulatus Squalene Monoxygenase and Oxidosqualene Cyclase Reconstitution and Activity

Squalene monoxygenase activity was reconstituted following the addition of spinach ferredoxin and ferredoxin reductase as electron donor partners and NADPH as a source of reducing equivalent. Due to the ease with which recombinant squalene monoxygenase lost its FAD prosthetic group during purification procedures, exogenous FAD was added to the purified protein preparation at 1:1 molar ratio. The recombinant squalene monoxygenase-catalyzed epoxidation of squalene was detectable by a peak in the GC trace, which had a retention time of 23.5 min compared to the retention time of squalene, 32 min (fig. 4A). The peak at 23.5 min had molecular ion at 426 m/z and verified as oxidosqualene, compared to squalene, which had

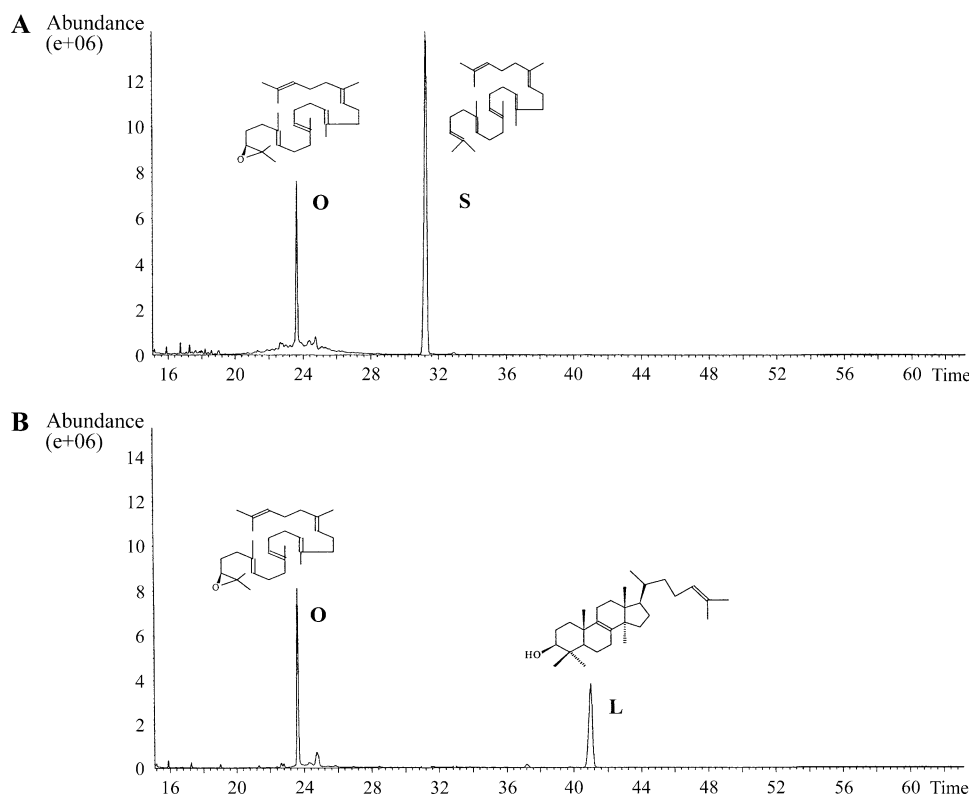


FIG. 4.—Gas chromatogram of the extracted sterols following reconstitution of *M. capsulatus* squalene monooxygenase and lanosterol synthase catalytic activity. Peaks can be identified as follows: S, squalene; O, (3*S*)-2,3-oxidosqualene; and L, lanosterol. The peak at 41.5 min gave a molecular ion at 498 in electron impact mass spectrometry, showing it to be lanosterol, and was identical to the fragmentation pattern for authentic purified lanosterol.

molecular ion at 410 m/z . In control experiments, the fragmentation pattern of the molecular ion of the pure oxidosqualene (Echelon, USA) was identical to the mass spectrometry fragmentation pattern observed for the product generated with purified *M. capsulatus* squalene monooxygenase. The activity was calculated to be 0.35 nmol oxidosqualene formed/min/nmol squalene monooxygenase, with no metabolism detected in the absence of enzyme or using boiled enzyme. Catalytic activity of *M. capsulatus* oxidosqualene cyclase was investigated using oxidosqualene as substrate. After 15-min incubation, the reconstitution reaction mixture was extracted using ethanolic potassium hydroxide, and the sterols were partitioned into hexane, silylated, and analyzed by gas chromatography and mass spectrometry (GC-MS). Following analysis the extracted reaction mixture contained two principal sterols (fig. 4B). A compound whose formation was dependent upon the presence of oxidosqualene cyclase had a retention time of 41.5 min and a molecular ion at 498 m/z . This compound was identified as the silylated lanosterol. In control experiments, the fragmentation pattern of the molecular ion of the silylated pure lanosterol was identical to the mass spectrometry fragmentation pattern observed for the product generated with purified *M. capsulatus* oxidosqualene cyclase. The activity was calculated to be 0.6 nmol lanosterol formed/min/nmol oxidosqualene cyclase, with no metabolism detected in the absence of enzyme or with boiled enzyme. As reported

previously for partially purified pig and rat lanosterol synthase (Dean et al. 1967; Moore and Schatzman 1992), there was no obvious need for organic coenzymes or cofactors in the cyclization reaction. This observation shows that this prokaryotic oxidosqualene cyclase can catalyze cyclization of oxidosqualene solely using functional electron-rich amino acid side chains within the active site of the protein molecule. Both the squalene monooxygenase and the oxidosqualene cyclase activities observed here are in the range reported before for other eukaryotic organisms (Yamamoto, Lin, and Bloch 1969; Laden, Tang, and Porter 2000). Further studies are underway to identify the endogenous squalene monooxygenase reductase partner and to fully characterize these activities.

Discussion

The evolution of sterol biosynthesis pathways is presumed to have occurred following the appearance of oxygen in the early Earth's atmosphere, after the Bacteria had become established. Most commonly, sterol biosynthesis is associated with eukaryotes, where an extended biosynthetic pathway exists involving over 20 genes/proteins. Each step presumably conferred a selective advantage, fixing each gene within the genome, and this enhanced fitness may be associated with increased osmotic or environmental robustness in primitive single-celled organisms. As discussed

above, the second enzymatic reaction to evolve was lanosterol or cycloartenol synthase, and the precise order of appearance of these pathways is open to conjecture. The complex cyclization events undertaken in either reaction are represented in the protists, with *Dictyostelium discoideum* following the cycloartenol route (Nes et al. 1990), whereas *Trypanosoma cruzi* synthesizes sterol via a lanosterol route (Buckner et al. 2003). Further prokaryotic and eukaryotic genome sequences will cast light on the root of both branches of sterol biosynthesis and suggest whether they had an origin in the prokaryotic era.

Interestingly, the oxidosqualene cyclase described here has closest homology to that of *G. obscuriglobus* and is located in a putative operon along with the first post-squalene enzyme, squalene monooxygenase, encoded by an open reading frame downstream of that for oxidosqualene cyclase. The reading frames overlap in the sequence 5' ATGA, with the ATG start codon for squalene monooxygenase within the same base sequence encoding the TGA termination triplet for oxidosqualene cyclase. During the course of this work, the presence of these adjacent genes in the *M. capsulatus* genome was also observed by others (Pearson, Budin, and Brocks 2004). In contrast to the genetic organization described for squalene monooxygenase and oxidosqualene cyclase, the gene encoding the next step of sterol biosynthesis in *M. capsulatus*, sterol 14 α -demethylase (*CYP51*), is genetically distant, being some 171 kb away on the chromosome (fig. 2). Obviously the presence of sterol gene clusters would facilitate horizontal transfer mechanisms, but the addition of the sterol 14 α -demethylase step may also have allowed further horizontal transfer and subsequent evolution of *CYP51*-like genes undertaking other gene functions in actinomycetes, e.g., in Streptomycetes and Mycobacteria (Bellamine et al. 1999; Lamb et al. 2002). No further genes of direct relevance to sterol biosynthesis could be identified at the loci examined. As the number of genomes increases, further refinements and insights will emerge and experimentation will be needed. This will be within both sterol producers and also in non-sterol-producing organisms, where putative gene products can show striking homology to known sterol biosynthetic enzymes, e.g., sterol C24-reductase homologues in *Coxiella burnetii* (personal observation).

Similar to eukaryotic lanosterol synthases, the *M. capsulatus* oxidosqualene cyclase contains many tyrosine and tryptophan residues that contain electron-rich side chains. *M. capsulatus* oxidosqualene cyclase possesses 3.8% tryptophan and 2.8% tyrosine residues compared with 2.3% and 5.5%, 3.1% and 4.5%, and 3.5% and 4.5% for *S. cerevisiae*, *Homo sapiens*, and *Arabidopsis thaliana*, respectively. Indeed, for all known and predicted lanosterol synthases from different kingdoms of life, the combined levels of tryptophan and tyrosine represent approximately 7% of all the amino acids from each gene product, indicating an essential role for such residues in catalysis (fig. 5). These findings support the hypothesis proposed by Shi, Buntel, and Griffin. (1994), that such electron-rich side chains are essential features of the cyclase active site where they direct the folding of oxidosqualene through stabilization of positively charged (C8 or C9 cation) intermediates during rearrangement. This was recently confirmed by the

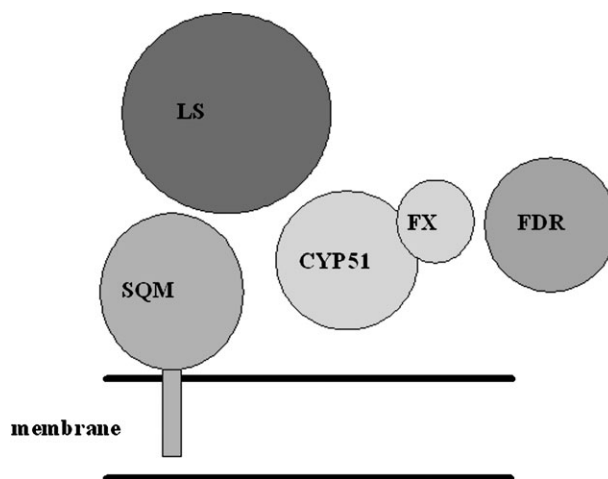


FIG. 5.—Proposed model of protein-protein interactions for the 3 postsqualene sterol biosynthetic enzymes from *Methylococcus capsulatus*.

structural resolution of human lanosterol synthase (Thoma et al. 2004).

Site-directed mutation studies have altered the cyclization products of a cycloartenol synthase so that different sterol products are produced (Wu and Griffin 2002). Whole-cell sterol analysis from the prokaryote *G. obscuriglobus* revealed 2 end products where the major product was parkeol with smaller quantities of lanosterol (Pearson, Budin, and Brocks 2004). We conclude the *M. capsulatus* oxidosqualene cyclase represents an important example of a prokaryotic protein using the lanosterol route to sterol synthesis. The only significant product detected in *in vitro* reactions was lanosterol, and that may be a reflection of the substrate specificity of the subsequent sterol 14-demethylase (*CYP51*) reaction. The importance of a sterol C8 double bond for activity by other sterol 14 α -demethylases has been indicated previously (Aoyama et al. 1989; Shyadehi et al. 1996), and this enzyme is the only cytochrome P450 family found in eukaryotes and prokaryotes.

As we speculate above, it may be possible that the soluble *M. capsulatus* sterol biosynthetic proteins may be positioned in a membrane location for biosynthesis via other scaffold proteins (tethers) associated with the bacterial membranes. Of interest in this regard was our discovery of the membrane-bound nature of the first postsqualene biosynthetic enzyme from *M. capsulatus*, squalene monooxygenase. This may be the initial membrane-associated protein of the sterol enzyme pathway complex (squalene monooxygenase, oxidosqualene cyclase, and lanosterol 14 α -demethylase) (fig. 6). We are identifying the appropriate electron transport system supporting *M. capsulatus* squalene monooxygenase, because these are not encoded within the putative operon for squalene monooxygenase and oxidosqualene cyclase synthesis, but are essential for activity in eukaryotes (Laden, Tang, and Porter 2000). Indeed, we have preliminary experimental evidence that a ferredoxin reductase within the *M. capsulatus* genome (fig. 2) can interact with and drive the catalytic activity of the *CYP51*-ferredoxin fusion protein (unpublished data).

In conclusion, although many bacteria produce hopanoids, and these are regarded as sterol surrogates, a small

number do produce true sterols. It is possible that the route to sterol biosynthesis was advantageous, because squalene monooxygenase provided a route to detoxify atmospheric oxygen, and then the evolution of oxidosqualene cyclase provided a molecule with superior qualities in membrane structure/function. Our experiments now confirm biochemically the presence of a postsqualene lanosterol biosynthetic enzyme complex within a prokaryotic sterol pathway in contrast to the lanosterol/parkeol synthase in *G. obscuribolus* and the cycloartenol synthase in *S. aurantiaca* (Bode et al. 2003). Further experiments on the enzymological properties of the *M. capsulatus* prokaryotic proteins are now possible.

Acknowledgments

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