Directed Evolution To Investigate Steric Control of Enzymatic Oxidosqualene Cyclization. An Isoleucine-to-Valine Mutation in Cycloartenol Synthase Allows Lanosterol and Parkeol Biosynthesis

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Oxidosqualene cyclases convert (*S*)-2,3-oxidosqualene to triterpene alcohols by protonation, cyclization, rearrangement, and elimination reactions. This catalytic approach has great plasticity and produces a large family of compounds; 83 different natural products are $C_{30}H_{50}O$ isomers structurally consistent with being enzymatically rearranged products of oxidosqualene.[1,2] Two rigorously conserved *Saccharomyces cerevisiae* lanosterol synthase residues (H146 and D456) have been shown by mutation to be essential,[3] and regions that contribute to product specificity between β-amyrin synthase and lupeol synthase have been mapped using chimeras.[4] The squalene-hopene cyclases are a related group of bacterial enzymes that initiate cyclization by olefin protonation.[5] The *Alicyclobacillus acidocaldarius* squalene-hopene cyclase[6] crystal structure[7,8] has been determined and used to identify active-site aromatic residues important for catalysis.[9,10]

If the deprotonation motifs in oxidosqualene cyclases were known, mutants that yield a variety of products could be constructed. Conversely, mutants with novel product specificities would reveal the amino acids that guide specific transformations by appropriately positioning active-site steric bulk and electron density. Reasoning that mutant cycloartenol synthases might be obtained that abstract the C8 proton of the lanosteryl cation to produce lanosterol (Figure 1), we selected spontaneously occurring cycloartenol synthase mutants that restored sterol-independent growth to a yeast lanosterol synthase mutant and thereby identified a cycloartenol synthase amino acid residue that contributes to product specificity.

Lanosterol is the initial cyclic intermediate in the biosynthesis of ergosterol, an essential fungal membrane component. SMY8 is a yeast lanosterol synthase deletion mutant[11] that requires ergosterol supplementation (20 mg/L). *Arabidopsis thaliana* cycloartenol synthase (CAS1)[12] was subcloned into pRS426GAL (made by subcloning the *GAL1* promoter[13] into pRS426[14]) and expressed in SMY8. The resultant strain uses its native pathway to biosynthesize oxidosqualene, which it converts to cycloartenol using the foreign enzyme. Because this mutant cannot convert cycloartenol to ergosterol, it remains dependent on exogenous sterol. This cycloartenol-producing strain was shaken at 30 °C in 500 mL of inducing medium (YPG+heme)[11] supplemented with trace amounts (0.2 mg/L) of ergosterol. The low ergosterol level limited growth so that after 24 h, the culture was only slightly turbid ($OD_{600} \approx 0.5$, ~20-fold less dense than a 20 mg/L ergosterol culture). A 5-mL aliquot of this culture was used to inoculate 500 mL of the same medium, and this process was iterated daily to allow more rapidly growing mutants that evolved lanosterol biosynthetic ability to dominate the culture. After 17 days, the culture reached a dramatically higher density ($OD_{600} \approx 10$), indicating that a spontaneous mutant arose with lower ergosterol requirements. A plasmid recovered from one colony complemented a yeast lanosterol synthase mutant, indicating that the plasmid conferred ergosterol-independent growth. The cycloartenol synthase gene in this plasmid contained two mutations: His158Gln and Ile481Val. Both single-mutant cycloartenol synthase genes were constructed and introduced into SMY8; only the Ile481Val derivative (CAS1Ile481Val) allowed ergosterol-independent growth.

CAS1Ile481Val converts oxidosqualene in vitro to a mixture of cycloartenol, lanosterol, and parkeol. A 3-L incubation (YPG+heme) provided 40 g of yeast, and a cell-free homogenate converted 200 mg of (±)-oxidosqualene to 70 mg of products.[15] The triterpene alcohol products were acetylated, and ^{1}H NMR analysis revealed three compounds including cycloartenyl acetate[16] and lanosteryl acetate.[17] A multiplet at 5.2 ppm indicated a third product containing a trisubstitued olefin distinct from that in the side chain. Preparative argentic TLC[18] provided three pure compounds, two of which were indistinguishable from authentic cycloartenyl acetate[12] and lanosteryl acetate[19] standards by ^{1}H NMR and GC-MS. The third product was shown to be parkeyl acetate by de novo characterization based on GC-MS, ^{1}H and ^{13}C NMR, COSYDEC, DEPT-135, HMBC, HSQC, and NOE difference spectra. GC analysis of the acetate mixture prior to argentic chromatography showed that CAS1Ile481Val makes cycloartenol (C19 deprotonation), lanosterol (C8 deprotonation), and parkeol (C11 deprotonation) in relative ratios of 52:26:22 (Figure 1). The wild-type cycloartenol synthase expressed similarly produced cycloartenol and parkeol (99:1), but did not produce detectable lanosterol (<0.1%).

A potential complication with this assay is that in vivo oxidosqualene biosynthesis in the expression host SMY8 allows cycloartenol, lanosterol, and parkeol production. Lanosterol is metabolized to ergosterol in vivo, and if cycloartenol or parkeol were metabolized more slowly, these compounds would accumulate and skew the product ratios observed. A yeast strain doubly mutant in lanosterol synthase and squalene synthase was constructed to block triterpene biosynthesis, permitting the

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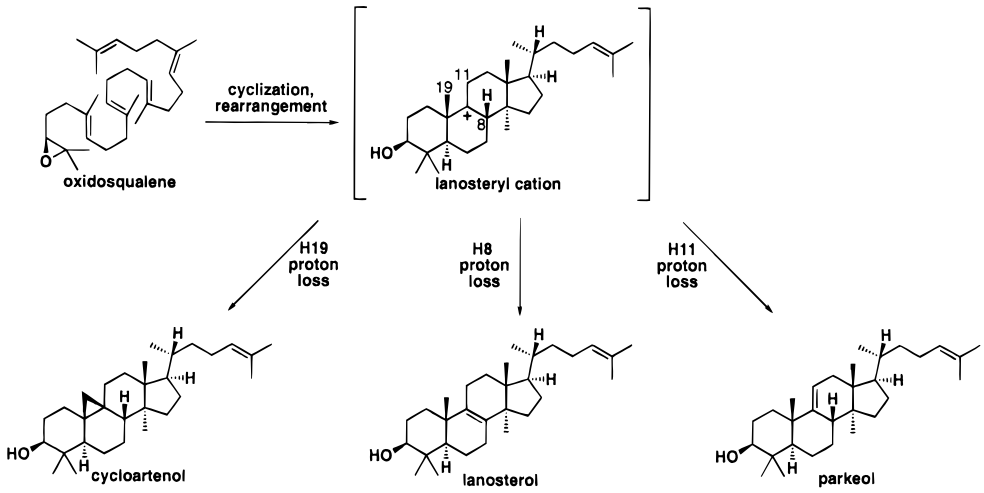

Figure 1. Oxidosqualene cyclization to cycloartenol, lanosterol, and parkeol. Cycloartenol, lanosterol, and parkeol are formed by distinct naturally occurring enzymes, each of which similarly protonates, cyclizes, and rearranges oxidosqualene to the lanosteryl cation, but abstracts a different proton to terminate the reaction. Cycloartenol results from ring closure and deprotonation from C19. Lanosterol is formed by deprotonating the lanosteryl cation from C8, and parkeol by deprotonation from C11. The cycloartenol synthase Ile841Val mutant cyclizes oxidosqualene to all three products.

rigorous analysis of in vitro oxidosqualene cyclization. The squalene synthase gene (*ERG9*) was deleted from yeast strain SMY4[11] by transforming a construct that replaced the genomic *ERG9* with the selectable marker *HIS3*. The resultant strain, LHY1, required squalene or ergosterol to live. The double mutant LHY2 (lacking *ERG9* and *ERG7*, encoding lanosterol synthase) was constructed by transforming LHY1 with a construct that replaced *ERG7* with the selectable marker *LEU2*. The *leu2* marker was recovered by integrating *hisG-URA3-hisG*[20] into the *LEU2* locus of the *ERG7* disruption to make LHY3 and selecting for spontaneously occurring *ura3* deletions with 5-fluoroorotic acid.[21] The resultant squalene synthase/lanosterol synthase double mutant was named LHY4 (*MAT***a** *erg7::leu2::hisG erg9::HIS3 hem1::TRP1 ura3-52 trp1-*Δ*63 leu2-3,112 his3-*Δ*200 ade2* Gal^+). CAS1Ile481Val expressed in LHY4 produced cycloartenol, lanosterol, and parkeol in relative ratios of 54:25:21, essentially the same as those observed in the lanosterol synthase single mutant. The LHY4 strain will be invaluable for expressing oxidosqualene cyclases when accumulating triterpene alcohol products in vivo could bias quantitation or cause toxicity.

CAS1Ile481 corresponds to active-site residue Asp374 in squalene-hopene cyclase[6] and is probably an active-site residue. Mutating isoleucine to valine replaces a *sec*-butyl side chain with a smaller isopropyl group, slightly enlarging the active site cavity. This steric change compromises the strict C19 deprotonation to cycloartenol and allows deprotonation at C8 and C11 to form lanosterol and parkeol, respectively. In native cycloartenol synthase, the critical methylene favors cyclopropyl formation relative to olefin formation, perhaps by forcing C19 and the still-unidentified active-site base into proximity or by shielding C8 and C11 from deprotonation. Conservation patterns support the importance of having steric bulk with the appropriate size and shape at this position, as isoleucine is strictly conserved in the known cycloartenol synthases.[12,22−24] The strict conservation of valine at the corresponding position in lanosterol synthases strongly indicates its relevance to lanosterol biosynthesis.[1] Mutagenesis experiments are in progress to identify additional residues that contribute to product specificity.

These experiments illuminate the forces that govern the evolution of enzymes and metabolic pathways. Fungi and animals cyclize oxidosqualene to the tetracyclic lanosterol, which can be metabolized to sterols without ring modification. Plants instead form the pentacyclic cycloartenol, and consequently require an additional enzyme (cyclopropyl isomerase) to open the cyclopropyl ring and form tetracyclic sterols.[25] It is difficult to rationalize on functional grounds why plants maintain the longer pathway because the sole established role of cycloartenol is sterol precursor, and plants can readily metabolize exogenous lanosterol to normal membrane sterols.[26] Furthermore, our induced evolution of lanosterol synthase required so little time or biomass that the Ile481Val mutation must arise frequently in the global pool of cycloartenol synthase genes. Plants may be locked into a local energy minimum with respect to sterol biosynthesis; their inability to exploit these mutations may result from difficulties in optimizing the Ile481Val mutant to abolish parkeol biosynthesis. Parkeol fed to tobacco is not demethylated to produce a sterol, but is instead oxidized to 24,25 epoxyparkeol.[27] Although acquiring the CAS1Ile481Val mutation could eventually lead a plant to the more efficient lanosterol route, in the short term it might divert carbon from biosynthesizing sterol to epoxytriterpenes. The decrease in sterol biosynthetic efficiency and appearance of an abnormal lipid might be expected to compromise the cellular membranes. Similar phenomena may govern the evolution of many metabolic pathways.

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Supporting Information Available: Enzyme incubation conditions, the silver chromatography procedure, details of triterpene acetate characterization by GC-MS and ^1H and ^{13}C NMR, and a 500 MHz ^1H spectrum of parkeol (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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