Communications to the Editor

A Tyrosine-to-Threonine Mutation Converts Cycloartenol Synthase to an Oxidosqualene Cyclase that Forms Lanosterol as Its Major Product

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Received April 17, 2000

The cationic rearrangement of oxidosqualene to cyclic triterpenes is catalyzed by the oxidosqualene cyclases, an enzyme family that produces more than 80 different naturally occurring triterpenes.[1] Experiments with modified cyclases have recently revealed structural features that control product formation. Chimeras that combine portions of β-amyrin synthase[2] and lupeol synthase[3] have been used to map catalytically relevant regions.[4] A directed evolution experiment designed to select randomly generated *Arabidopsis thaliana* cycloartenol synthase[5] mutants that produce lanosterol uncovered an Ile481Val mutant that forms 25% lanosterol and 21% parkeol in addition to cycloartenol.[6] *Saccharomyces cerevisiae* lanosterol synthase mutants with slightly greater steric bulk at the corresponding Val454 residue (Ile or Leu mutants) remained accurate lanosterol synthases, but decreased steric bulk caused significant production of the monocyclic triterpene achilleol in the Ala (5%) and Gly (15%) mutants.[7] We describe herein the discovery of a cycloartenol synthase residue that is key to the catalytic difference between cycloartenol synthase and lanosterol synthase.

We identified candidates for this role by examining conservation patterns between cycloartenol and lanosterol synthases. Cycloartenol synthases have been cloned and characterized from five plants[2,5,8] and a slime mold.[9] Lanosterol synthases are known from three fungi and two animals.[1] Anticipating that some active-site positions have one catalytic role in cycloartenol synthase and a different one in lanosterol synthase, we looked for positions at which cycloartenol synthase and lanosterol synthase residues are conserved differently from one another. In addition to the previously studied Ile481 residue,[6] four positions fit the criteria: Tyr410(Thr), Gly488(Ala), Phe717(Trp), and Met731(Ala).[10]

We generated cycloartenol synthase derivatives with each of these residues changed to the corresponding lanosterol synthase residue (Tyr410Thr, Gly488Ala, Phe717Trp, and Met731Ala). Initial studies used protein expressed with the vector pRS305GAL[11] in the lanosterol synthase mutant yeast strain SMY8.[11] Expression and in vitro incubation with racemic oxidosqualene as described previously[7] provided triterpene alcohol products, which were acetylated and identified by GC, GC−MS, and NMR. The Gly488, Phe717, and Met731 mutants remained cycloartenol synthases, but the GC trace of the acetylated Tyr410Thr product showed two signals consistent with lanosteryl acetate and parkeyl acetate rather than cycloartenyl acetate. ^{1}H NMR analysis confirmed the presence of these two compounds but also revealed substantial amounts of an additional product distinct from known triterpene acetates.

To further characterize and quantitate these products, we expressed the mutant enzyme in the yeast strain LHY4,[6] which lacks both lanosterol synthase and squalene synthase.[12] Incubation with racemic oxidosqualene, acetylation of the triterpene alcohol products, and argentation chromatography[13] provided three triterpene acetates. Lanosteryl acetate and parkeyl acetate were identified by comparing ^{1}H and ^{13}C NMR spectra to those of authentic standards.[6,14] HRMS and NMR studies (^{1}H, HSQC, COSYDEC, ^{13}C, DEPT, and NOE difference spectra) of the third acetate established the novel triterpene as 9β-lanosta-7,24-dien-3β-ol (9β-Δ^7-lanosterol). GC did not completely resolve the Δ^8 and 9β-Δ^7 isomers, which were consequently quantitated by analyzing the ^{1}H NMR spectrum of the crude triterpene acetates. Mechanisms of the relevant oxidosqualene cyclization reactions are shown in Scheme 1. Neither 9β-Δ^7-lanosterol nor obvious metabolites of this compound have been described. The known natural Δ^7-sterols have 9α stereochemistry (or are $\Delta^{7,9(11)}$ dienes) and are probably lanosterol metabolites.[15]

The Tyr410Thr Ile481Val double mutant was constructed and analyzed similarly to examine potential synergistic effects with the previously characterized Ile481Val mutation.[6] The single mutants (Table 1) produced lanosterol accompanied by parkeol and either cycloartenol (Ile481Val) or 9β-Δ^7-lanosterol (Tyr410Thr). The double mutant produced a higher proportion of lanosterol than did the single mutants (Table 1).

The positions relative to the active site of the *S. cerevisiae* residues have been predicted[9] using the *A. acidocaldarius* squalene-hopene cyclase[16] crystal structure[17] as a guide. The

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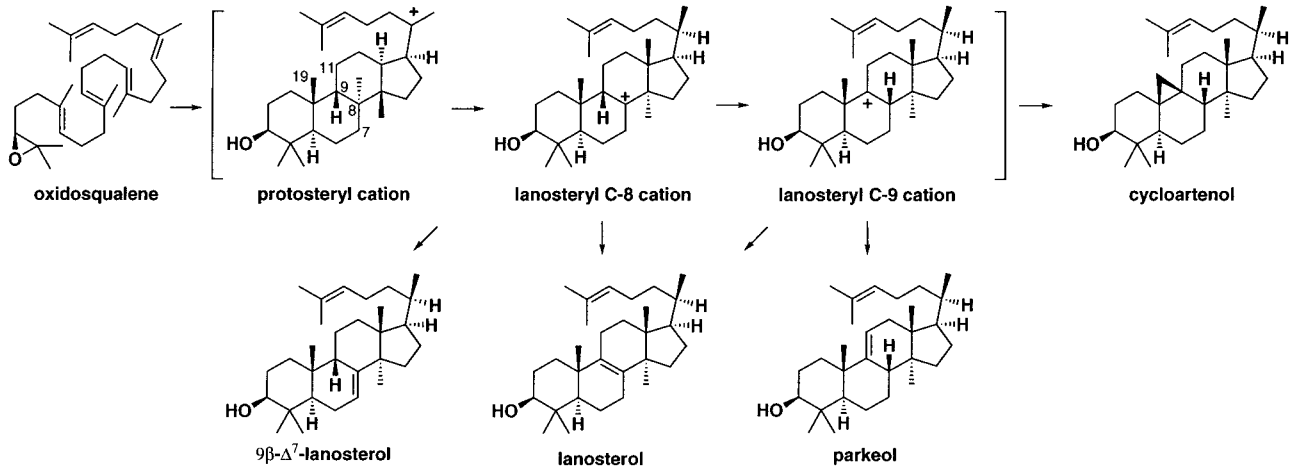
10.1021/ja0013226 CCC: $19.00
Published on Web 06/28/2000

Scheme 1. Mechanisms for Forming Cycloartenol, Parkeol, Lanosterol, and 9β-Δ^7-Lanosterol[a]



[a] Oxidosqualene is cyclized to the protosteryl cation, which undergoes rearrangement and deprotonation reactions to the $C_{30}H_{50}O$ compounds shown. Cycloartenol and parkeol are easily rationalized as coming from the C-9 cation, whereas the C-8 cation seems a more likely progenitor of 9β-Δ^7-lanosterol. Lanosterol could arise from either intermediate cation.

Table 1. Percent Product Composition of *A. thaliana* Cycloartenol Synthase Mutants[a]

mutation	cycloartenol	lanosterol	parkeol	9β-Δ^7−lanosterol
none	99	0[b]	0.6	0[b]
Ile481Val	56	24	20	0[b]
Tyr410Thr	0[b]	65	2	33
Tyr410Thr Ile481Val	0[b]	75	0.6	24

[a] Crude LHY4 sterol isolates were analyzed by ^{1}H NMR (500 MHz), to quantitate minor components. GC quantitation of triterpene acetates derived from the native enzyme and Ile481Val mutant gave comparable results (±1%).[6] The δ 0.5−1.1 region of the NMR spectra also showed additional minor singlets that may represent unidentified triterpene products (generally corresponding to ≤1% of total triterpenes). All mutants remained effective catalysts, generating >0.5 mg products/mL homogenate. [b] Not detected; detection limit ≤0.3%.

catalytically important residues Tyr410 and Ile481 correspond to *S. cerevisiae* residues predicted to be in or near the active site, whereas Gly488, Phe717, and Met731 correspond to residues >8 Å from the ligand. The synergistic effect of the 410 and 481 mutations (*A. thaliana* cycloartenol synthase numbering) indicates that both positions participate in specific formation of lanosterol. The Tyr410Thr mutation abolishes cycloartenol formation, suggesting that cyclopropane formation requires some substructure of tyrosine. The positions of the Tyr and Thr hydroxyl groups or electronic differences between them could account for the catalytic difference. Alternatively, the Tyr410 π-electrons could facilitate cycloartenol formation by stabilizing the C-9 cation.[18] Lanosterol, cycloartenol, and parkeol are conceivable deprotonation products from that cationic intermediate. However, 9β-Δ^7-lanosterol is more readily rationalized as arising from a C-8 cation (Scheme 1),[19] and the Tyr410Thr mutant might preferentially stabilize that cation, or might fail to promote the hydride shift from C-9 to C-8.

Positions corresponding to Tyr410 also exhibit a striking conservation pattern in the characterized enzymes that form the dammarenyl cation intermediate (lupeol synthase[3,20] and β-amyrin synthase[2,21]). Tyr410 may be located near the B/C ring fusion; its mutations affect deprotonation in that region. Tyr410 is preceded by Gly and followed by Asn in all known cycloartenol and lanosterol synthases (which utilize the protosteryl cation intermediate) to comprise GlyTyrAsn and GlyThrAsn motifs. Lupeol synthases and β-amyrin synthase have the two residues SerPhe and a single amino acid deletion at the corresponding positions. The protosteryl and dammarenyl cations have inverted stereochemistries at the B/C ring fusion, and the SerPhe motif in lupeol synthase and β-amyrin synthase might facilitate dammarenyl cation formation or play some other role specific to nonsteroidal triterpene formation.

In conclusion, these modifications of an oxidosqualene cyclase reflect how readily relatively minor mutations can alter product structure. The novel 9β-Δ^7-lanosterol structure highlights the potential of mutant oxidosqualene cyclases to generate new compounds. This catalytic plasticity of terpene cyclases evidently facilitated the evolution of an enzyme family that produces a vast diversity of triterpene skeletons.

Acknowledgment. We thank Jihai Pang for mass spectral analyses and Elizabeth A. Hart for samples of CAS1 and CAS1Ile481Val products and for valuable comments on the manuscript. This research was funded by the NIH (AI41598) and the Robert A. Welch Foundation (C-1323). J.B.R.H was supported by NIH Biotech Training Grant T32 GM08362. W.K.W. was supported by NIH Grant HL49122.

Supporting Information Available: Details of triterpene acetate characterization by NMR, including ^{1}H and ^{13}C NMR spectra of 9β-Δ^7-lanosterol and tables of chemical shifts (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

JA0013226

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