Enzymatic Formation of Multiple Triterpenes by Mutation of Tyrosine 510 of the Oxidosqualene-Lanosterol Cyclase from *Saccharomyces cerevisiae*

Tung-Kung Wu* and Cheng-Hsiang Chang[a]

Triterpene cyclases constitute a family of enzymes that catalyze diverse and complex carbocationic cyclization/rearrangement reactions of squalene and (3*S*)-2,3-oxidosqualene (OS) to generate a distinct array of sterols and triterpenes.[1,2] A major determinant for the triterpenoid diversity is believed to be the precise control of conformation between substrate and enzyme, as well as the position of the carbocation intermediate formation. For example, both oxidosqualene-lanosterol cyclase (ERG7, EC 5.4.99.7) and oxidosqualene-cycloartenol synthase (CAS, EC 5.4.99.8) bind oxidosqualene in a chair–boat–chair conformation, initiate and propagate cyclization to form a protosteryl cation, and then promote 1,2-shifts of hydride and methyl groups to the lanosteryl C8 cation. The lanosterol formation is accomplished through the final deprotonation, abstracting a proton originally at C9 or after a hydride shift from C9 to C8. Cycloartenol is formed after a hydride shift from C9 to C8, followed by 9β,19-cyclopropane ring closure.

Elegant molecular-genetic and bioorganic investigations have recently identified several amino acid residues that are critical in probing putative active sites and determining prod-

[a] *Prof. Dr. T.-K. Wu, C.-H. Chang*
Department of Biological Science and Technology
Center for Interdisciplinary Molecular Science
National Chiao Tung University
300, Hsin-Chu, Taiwan (Republic of China)
Fax: (+886) 35-725-700
E-mail: tkwml@mail.nctu.edu.tw

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uct profiles in triterpene cyclase enzymes.[3–16] For example, the Matsuda group used site-directed mutagenesis and directed evolution to probe putative active-site residues within *Saccharomyces cerevisiae* ERG7 and *Arabidopsis thaliana* CAS, and demonstrated that Val454 of *S. cerevisiae* ERG7 and Ile481 of *A. thaliana* CAS are critical in determining cyclization-product profiles during the oxidosqualene cyclization cascade.[3,7,9,11,14] Further mutagenesis of ERG7^{Val454} to various residues (Ala and Gly) resulted in the production of an additional truncated monocyclic achilleol A other than the native enzymatic product, lanosterol; this indicated the steric effect of this residue on the catalysis of cyclization and/or deprotonation steps.[7] We recently identified five single-point mutations within the *A. thaliana* CAS enzyme that lead to the production of lanosterol and support the growth of an ERG7-deficient *S. cerevisiae erg7* knockout strain.[15] As part of this study, we also determined that a Tyr532His mutation in *A. thaliana* CAS produces both lonosterol and the monocyclic product achilleol A.

Given the changes in product specificity observed with the *A. thaliana* CAS Tyr532His mutant, we hypothesized that the corresponding residue in *S. cerevisiae* ERG7, Tyr510, as well as neighboring residues would also be important for controlling product specificity. Accordingly, amino acid residues 509–513 (TYEKI) of *S. cerevisiae* ERG7 were subjected to alanine-scanning mutagenesis, in vivo functional studies, and in vitro product analysis to determine the effect on the cyclization/rearrangement mechanism. As it has been observed that residue Tyr510 is conservatively different between ERG7/CAS and β-amyrin/lupeol synthase (data not shown), an additional Tyr510Trp substitution was carried out in order to investigate the possible effect on product diversity. To examine the effect of different electronic environments on cyclase function, the Tyr510Lys mutant was also prepared. We now report the identification of an oxidosqualene-lanosterol cyclase residue, Tyr510, that failed to complement cyclase-deficiency when mutated to tryptophan or lysine, but which maintained yeast viability when substituted with alanine. Moreover, the possible functional roles of this residue were elucidated by isolating and structurally characterizing incompletely cyclized and alternatively deprotonated products from the mutants.

The pRS314-derived ERG7-mutated plasmids were transformed into an ERG7 knockout yeast haploid strain CBY57[pZS11], bearing a genomic *erg7*-disrupted gene, and a pZS11 plasmid with a wild-type *S. cerevisiae* cyclase gene, and then plasmid shuffle-selected for their ability to complement the ERG7 deficiency. Yeast transformants were selected for growth on SD + Ade + Lys + His plates, and then reselected on SD + Ade + Lys + His + Ura + 5-fluoroorotic acid (5-FOA) plates to elucidate the complementation effects. In addition, plasmids that failed to complement the cell viability were isolated and sequenced to confirm the identity of the mutations. Among the various mutants, all of the alanine-scanning mutations, including Tyr510Ala, produced colonies upon counterselection for pZS11 with 5-FOA. In contrast, the Tyr510Lys and Tyr510Trp mutations failed to complement the ERG7 disruption. This finding suggests that different amino acid substitutions on Tyr510 might affect expression and/or stability of *S. cerevisiae* ERG7, might abolish all catalytic activity, or might alter cyclase product specificity to non-lanosterol products.

To elucidate the possible catalytic or structural role of the Tyr510Ala mutation, an 8 L culture (65 g of yeast cells) was collected, and the nonsaponifiable lipid (NSL) extract was prepared to analyze the product profiles. Chromatographic purification revealed a major product that was indistinguishable from lanosterol when examined by thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC). In addition, a significant amount of a second NSL, migrating between oxidosqualene and lanosterol, was also observed. This compound was later characterized as the monocyclic triterpene achilleol A by comparison with authentic GC-MS and NMR data.[15] Further GC-MS-based product analysis of the lanosterol-positioned product revealed two compounds with identical molecular mass ($m/z = 426$). The product assignments of these two compounds were confirmed by ^{1}H and ^{13}C NMR analyses of intact and acetylated product mixture. Distinct ^{1}H NMR chemical shifts for both lanosterol ($\delta = 0.687$ and 0.810 ppm) and parkeol ($\delta = 0.637$, 0.729, and 5.225 ppm) methyl groups and vinyl protons were observed when compared with data available in the literature.[9,17] The ^{13}C NMR spectra of the acetylated products also displayed all 32 lanosteryl acetate and parkeyl acetate signals, thus confirming the identities of lanosterol and parkeol. GC-MS analyses of these compounds showed that the NSL extract of *S. cerevisiae* ERG7^{Y510A} mutant accumulated achilleol A, lanosterol, and parkeol in a 27:39:34 ratio. By contrast, neither 9β-Δ^7-lanosterol nor any other signal with a mass consistent with oxidosqualene cyclization products could be detected.

To unambiguously determine the functional role of Y510K and Y510W mutations in ERG7 activity, the ERG7^{Y510K}- and ERG7^{Y510W}-mutated plasmids were transformed into a new cyclase-deficient *S. cerevisiae* TKW14 strain, a CBY57-derived HEM1 ERG7 double-knockout strain that bears genomic *erg7*- and *hem1*-disrupted genes, eliminates the pZS11 plasmid, and maintains cell viability through the uptake of ergosterol from the medium. As expected, the recombinant mutants bearing Y510K and Y510W mutations failed to maintain cell viability in the absence of ergosterol; this supports the theory that the above-mentioned mutations are unable to complement the ERG7 disruption. Both mutants were next grown in liquid media containing ergosterol, and harvested. Preliminary NSL-extract result showed that *S. cerevisiae* ERG7^{Y510K} and ERG7^{Y510W} are less efficient than native ERG7, generating $\sim 0.2\ mg\,L^{-1}$ culture (~2% the yield from native enzyme). The TLC and HPLC results from the nonsaponifiable lipid extracts showed that no lanosterol-positioned product was observed; this demonstrated the disruption of ERG7 activity. In addition, two non-lanosterol products that migrated between oxidosqualene and lanosterol were detected. Distinct ^{1}H NMR chemical shifts for achilleol A were observed, as previously described.[15] In addition, a multiplet at 5.216 ppm indicated a product containing a trisubstituted olefin distinct from that in the side chain. The ^{1}H and ^{13}C NMR as well as GC-MS analyses confirmed the identity of this compound as camelliol C, showing spectra consistent with data available in the literature.[18] ^{1}H NMR analysis of a

partially purified sample showed that ERG7^{Y510K} and ERG7^{Y510W} mutants make achilleol A and camelliol C in ratios of 86:14 and 96:4, respectively. The production of camelliol C in ERG7 of *S. cerevisiae* has not been reported before. The mechanisms of the relevant oxidosqualene cyclization/rearrangement reactions are shown in Scheme 1.

The Tyr510 of *S. cerevisiae* ERG7 corresponds to Tyr420 in *A. acidocaldarius* SHC and to Tyr532 in *A. thaliana* CAS. The residue has been previously mutated to phenylalanine and shown viability when transformed in an ergosterol-free medium.[19] However, no product isolation and characterization to investigate the catalytic function of this residue was carried out. On the other hand, the introduction of various mutations at Tyr420 in *A. acidocaldarius* SHC led to the production of several abortive cyclization products.[6,20]

A homology model of the *S. cerevisiae* ERG7 was created by using the Insight II Homology program with the X-ray structure of 2-azasqualene-bound SHC from *A. acidocaldarius* as the template.[16] Good agreements in the distribution of secondary structure, stereochemical quality, and 3D profile, as well as accordance with the human oxidosqualene-lanosterol cyclase (OSC) model further support the idea that *A. acidocaldarius* SHC is an appropriate template for homology modeling of various oxidosqualene-cyclases in general.[16,21] The model revealed that Tyr510 lies adjacent to the catalytic Val454 and Asp456 in ERG7 (Figure 1), as in the human OSC homology model, where Val454 influences B-ring formation but not deprotonation, and Asp456 initiates catalysis.[7,14,16,21] The observed hydroxyl group

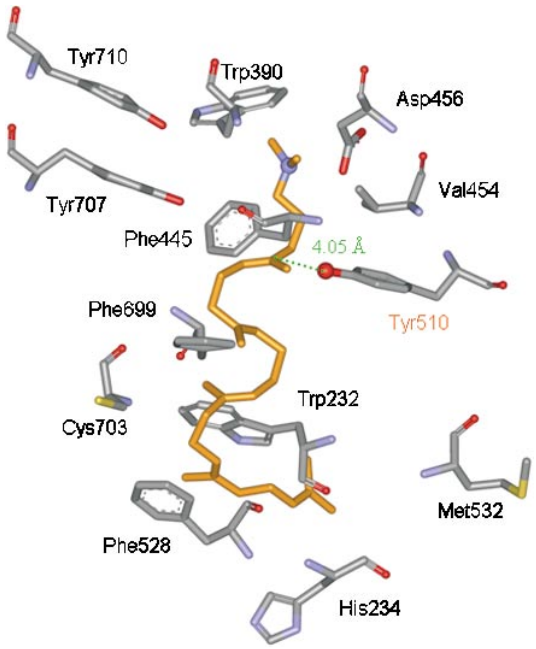
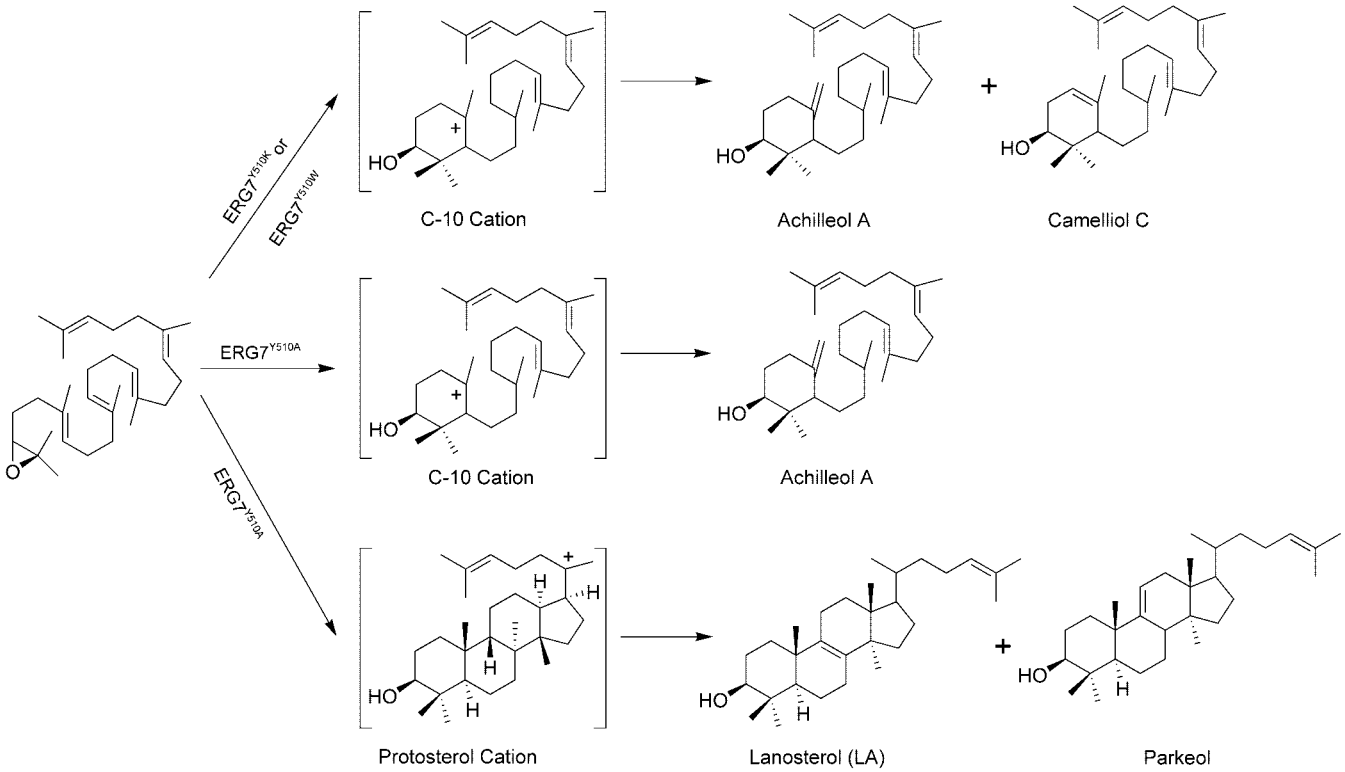


Figure 1. *Local views of the homology modeled* S. cerevisiae *ERG7 structure, based on the 2-azasqualene-bound SHC X-ray structure and determined by using the Insight II Homology program. Putative active site residues (stick representation) participating in the active site formation of modeled ERG7 structures are included. (2-azasqualene, orange; tertiary amine of 2-azasqualene, light blue; phenolic oxygen of Tyr510, red; dotted line and distance between C6 of 2-azasqualene and phenolic oxygen of Tyr510, green).*



Scheme 1. *Oxidosqualene-lanosterol cyclase Tyr510 mutants from* Saccharomyces cerevisiae *convert oxidosqualene to a variety of monocyclic and deprotonated structures including achilleol A, camelliol C, lanosterol, and parkeol.*

of Tyr510, derived from homology modeling of *S. cerevisiae* ERG7, was found at a distance of about 4.0 Å to the C10 cation of lanosterol; this distance agrees with the dipoles for observed aromatic amino acid residues at distances of 3.5 to 5.5 Å in the 2-azasqualene-bound SHC complex.[16] Therefore, its mutation to alanine may affect cyclization to achilleol A as well as deprotonation to lanosterol and parkeol, possibly through partial disruption of transient dipole interactions between carbocationic intermediates and the hydroxyl group of the aromatic ring of Tyr510. Similarly, an Y510W-mutated ERG7 homology model showed that the distance between the C6 of 2-azasqualene and the indole side chain of Trp was reduced to about 2.3 Å (data not shown) when the phenolic ring of Tyr was replaced with the indole ring of Trp. The models suggest that the bulky indole side chain might interfere with the progression of cyclization beyond the monocyclic stage, whereas the lysyl side chain may be unable to provide transition-state stabilization for such progression. Accordingly, a basic amino acid for abstracting a proton from Me-25 of achilleol A or CH_2-1 of camelliol C might be located proximal to the Tyr510 residue, and substitution of Tyr510 with a bulky Trp or Lys might cause enzyme perturbation or erroneous folding of OS and result in different ratios of premature cyclization products. However, the exact reason for the higher accumulation of achilleol A over that of camelliol C, and the production of achilleol A whenever camelliol C is produced, remain unclear.

In summary, the identification of the relevant residue in both ERG7 and CAS enzymes that is important for cyclase activity reveals the importance of this residue in controlling the catalytic or structure–function relationships of (oxido)squalene cyclases during the (oxido)squalene cyclization/rearrangement cascade. In addition, this is the first report that a single mutation in the ERG7 of *S. cerevisiae* can produce both achilleol A and camelliol C. Furthermore, the formation of various incomplete cyclization products and parkeol in addition to lanosterol in the Tyr510 mutant indicates that this residue might influence functions both in facilitating tetracyclic formation and stabilizing the lanosteryl C8/C9 cation for deprotonation. Finally, these experiments reflect how minor mutations can alter product specificity and highlight the potential for an increased diversity of triterpene skeletons through molecular evolution and metabolic engineering of the enzyme family of cyclase.

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