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Protein Plasticity: A Single Amino Acid Substitution in the *Saccharomyces cerevisiae* Oxidosqualene−Lanosterol Cyclase Generates Protosta-13(17),24-dien-3β-ol, a Rearrangement Product

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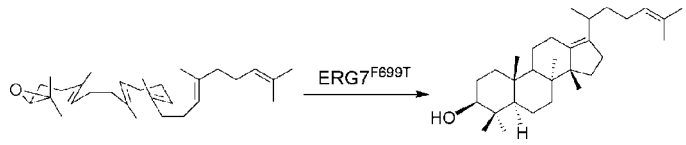
ABSTRACT



To provide insights into the structure−function relationships of oxidosqualene−lanosterol cyclase (ERG7) from *Saccharomyces cerevisiae*, the Phe699 was exchanged against hydrophilic polar uncharged residues Ser, Thr, Cys, Gln, and Tyr to characterize its product profile and functional role in ERG7 activity. Among the substitutions, only the ERG7^{F699T} mutant produced novel protosta-13(17),24-dien-3β-ol as the sole truncated rearrangement product. The results suggest that Phe699Thr mutation is likely to affect the C-17 cation stabilization during the rearrangement process.

Protein redesign with increased thermostability, altered binding specificity or affinity, enhanced enzymatic activity, or directed product specificity remains a difficult challenge for protein scientists despite some limited success.[1] Factors that may affect protein redesign include protein flexibility and dynamics, mutation-induced protein folding for substrate accommodation, binding interaction between transition state and key active site functional groups, and release of products. Identification of important plasticity residues in the target proteins with diverse product profiles is the first step in protein redesign to acquire new or higher specificity and activity.[1e,g] Yoshikuni et al. reported active-site saturation mutagenesis of plasticity residues from a sesquiterpene synthase, γ-humulene sesquiterpene synthase. This yielded mutant synthases with narrower product specificity and some with lower activities.[1e] To elucidate plasticity residues involved in the oxidosqualene cyclization/rearrangement reaction cascade, we applied homology modeling coupled with site-directed mutagenesis and product characterization. The expected results would decipher the structure−function relationships of the oxidosqualene cyclases and obtain protein with tailored product specificity or diversity. In this study, we describe an oxidosqualene cyclase mutant that terminates the oxidosqualene cyclization/rearrangement cascade at a

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truncated rearrangement step and generates an aberrant product, protosta-13(17),24-dien-3β-ol (**3**).

Oxidosqualene cyclases catalyze the enzymatic conversion of acyclic (3*S*)-2,3-oxidosqualene (**OS, 1**) into diverse polycyclic sterols and triterpenoids with remarkable regio- and stereospecificity.[2] Product specificity and diversity are species dependent and mainly controlled by the prefolded substrate conformation within the active-site geometry of the enzyme, as well as the molecular interaction between the carbocation intermediate and the functional groups of the amino acid residues and subsequent deprotonation. In fungi and mammals, oxidosqualene−lanosterol cyclase catalyzes the cationic cyclization/rearrangement reactions of **OS** into lanosterol (**2**). The reaction cascade is proposed to be initiated by the protonation and cleavage of an oxirane ring, followed by consecutive carbocation-mediated tetracyclic ring annulation and hydride/methyl group migration. The reaction is terminated by deprotonation through removal of a proton or addition of water.[3,4]

We previously established that Trp232, His234, Phe445, and Tyr510 are catalytically important residues in *Saccharomyces cerevisiae* oxidosqualene−lanosterol cyclase (ERG7) that influence the cyclization/rearrangement cascade and generate a diverse product profile.[3] These residues occupy part of the ceiling of the active site cavity, are spatially close to one another, but differentially affect deprotonation positions when substituted with different amino acid residues. His234 and Tyr510 are conserved in all lanosterol cyclases and cycloartenol synthases. However, the corresponding residues in β-amyrin synthases and lupeol synthases are Tyr and Trp, respectively. Subtle changes in the His234 catalytic environment have electronic effects on the intrinsic His234/Tyr510 H-bonding network and could sterically alter the active-site cavity structure to generate diverse product profiles ranging from monocyclic, tricyclic, tetracyclic, truncated hydride shift, and altered deprotonation products.[3c,e] The Trp232 affects lanosterol formation through steric interactions with C-11/C-12 and π-electron density. Substitution of Trp232 with nonpolar hydrophobic side chains such as Gly, Ala, Val, and Ile enlarged the cavity of the active site. This subsequently impaired the His234/Tyr510 catalytic base dyad H-bonding network, which resulted in the production of altered deprotonation products.[3d] Phe445 is spatially above the molecular plane of the substrate and proximal to both the B/C ring fusion and the C-8/C-14 positions. Polar side chain substitutions at the Phe445 position specifically generated truncated tricyclic and altered deprotonation products including (13α*H*)-isomalabarica-14(26),17*E*,21-trien-3β-ol, 9β-lanosta-7,24-dien-3β-ol, parkeol, and lanosterol.[3f]

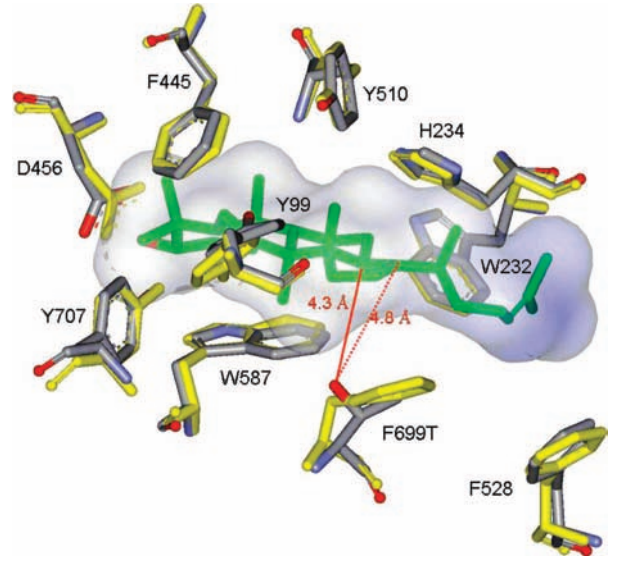

Figure 1. Homology structural model for the oxidosqualene−lanosterol cyclase active sites from *S. cerevisiae*, complexed with protosteryl C-17 cation. Superposition of the wild-type ERG7 (yellow) with the ERG7^{F699T} (residue color). Dotted line and distance between C-17 position of protosteryl C-17 cation and hydroxyl group of Thr699. Solid line and distance between C-13 position of protosteryl C-17 cation and hydroxyl group of Thr699.

Phe699 of ERG7 is spatially located on the other side wall of the active site cavity, exhibiting different spatial orientation relative to that of His234 (Figure 1). For human OSC, Thoma et al. show that His232 and Phe696 (which correspond to His234 and Phe699 of *S. cerevisiae* ERG7, respectively) are positioned near the C-13/C-20 positions of lanosterol and for π-cation interaction with the C-13 anti-Markovnikov tricyclic cation, which is created from the Markovnikov tricyclic C-14 cation (lanosterol numbering).[5] Careful examination of the homology model suggested that the His234 position should affect skeletal rearrangement on Me-14β→Me-13β 1,2-methyl group shift. In contrast, the Phe699 has the π-electron density positioned below the molecular plane of the substrate, as well as near the D-ring and the exocyclic terminal hydrocarbon side chain junction, and may affect either the H-17α→20α or H-13α→17α 1,2-hydride shifts. However, no product isolation and characterization was performed for Phe699 of ERG7 enzyme to substantiate its effect on catalysis. Moreover, because ERG7^{F699} exhibits spatial correlation to D-ring/exocyclic terminal junction, the disruption in lanosterol production was expected for the ERG7^{F699} mutation than that in ERG7^{F699} wild-

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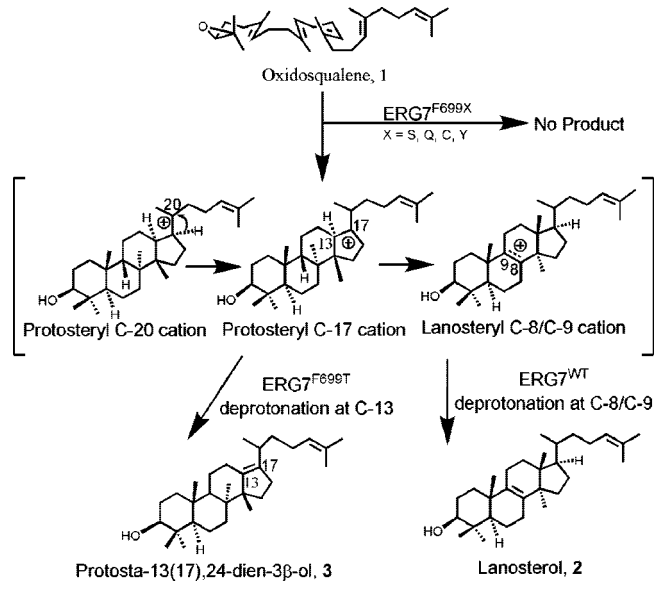
type. Further, high numbers of aromatic residues conserved in the oxidosqualene cyclases have been implicated in the correct folding of the π-electron-rich, hydrophobic substrate through $\pi-\pi$ interactions, which rigidly control the stereochemistry of the enzymatic reaction to lanosterol. Substitution of Phe699 with hydrophilic polar uncharged residues might disrupt the predominantly hydrophobic nature of the active site cavity resulting in an equilibrium shift from protosteryl cation to lanosteryl C-8/C-9 cation for lanosterol formation. It could also act as a basic residue to accept a proton in the specific deprotonation of the protosteryl C-17 cation that terminates catalysis. Thus, Phe699 was mutated to polar uncharged amino acid residues such as serine, threonine, cysteine, glutamine, and tyrosine to investigate their effects on catalysis or product specificity/diversity.

The ERG7^{F699S}, ERG7^{F699T}, ERG7^{F699C}, ERG7^{F699Q}, and ERG7^{F699Y} mutants were first expressed in a cyclase-deficient *S. cerevisiae* strain, TKW14, where the *erg7* and *hem1* genes are disrupted.[3a,b,6] Cells maintain viability through the uptake of the exogenous ergosterol, or they are complemented by the ERG7 activity expressed from the mutated plasmids. The genetic selection results showed that all of the mutations resulted in nonviable mutants, except for the ERG7^{F699T} mutation, which suggests that the Phe699 has a crucial role for the catalysis. The above-mentioned mutants were then expressed in the yeast oxidosqualene−lanosterol cyclase/lanosterol C-14 demethylase (ERG7/ERG11) double mutant strain YTL4.[7] The ERG7 deletion abolishes background cyclase activity, and the ERG11 deletion prevents the metabolic flux of lanosterol produced by the ERG7 mutant into the downstream sterol biosynthesis and consequently ensures the lack of endogenous ergosterol. The ERG7^{F699T} mutant showed similar growth rate to that of the wild type cells. A novel compound was isolated following nonsaponifiable lipid (NSL) extraction and $AgNO_3$-impregnated silica gel column separation of the product profile. This compound had a molecular mass of 426 Da and was isolated with stringent specificity (>99.8%) from the ERG7F699T mutant. Conversely, neither lanosterol nor the new product was isolated with the ERG7^{F699S}, ERG7^{F699C}, ERG7^{F699Q}, and ERG7^{F699Y} mutants. The new novel compound was characterized with NMR (^{1}H, ^{13}C NMR, DEPT, ^{1}H−^{1}H COSY, HMQC, HMBC, and NOE) and demonstrated to be protosta-13(17),24-dien-3β-ol based on the following data. The ^{1}H NMR spectra showed one olefinic proton (δ 5.044), two vinylic methyl signals (δ 1.639, 1.531), five methyl singlets (δ 1.029, 0.949, 0.935, 0.911, 0.775), and one methyl doublet (δ 0.925, d, J = 6.9 Hz, 3H). The 150 MHz ^{13}C NMR spectrum revealed the presence of one tertiary−quaternary (δ 124.96, 130.90) and one quaternary−quaternary substituted double bond (δ 135.23, 138.98). The HMQC spectrum showed that the methyl doublet protons at δ 0.925 were attached to the carbon at δ 20.05, the methine proton at δ 2.432 was attached to the carbon at δ 31.56 (C-20), while the methylene protons at δ 2.336 and 1.779 were attached to the carbon at δ 23.24 (C-12). In the HMBC spectrum, the δ 2.432 methine proton is coupled by ^2J to carbons at δ 135.23 (C-17), 35.64 (C-22), and 20.05 (C-21), as well as by ^3J to carbons at δ 138.98 (C-13), 28.65 (C-16), and 26.32 (C-23). The HMBC also established that the tertiary vinylic proton (δ 5.044) was coupled by ^2J to carbons at δ 130.94 (C-25) and 26.32 (C-23), as well as by ^3J connectivity to carbons at δ 25.71 (C-26), δ 17.58 (C-27), and 35.64 (C-22). Spectroscopic results obtained from the HMQC and HMBC correlations establish key structural features of the two double bonds located between C-13 and C-17 and between C-24 and C-25. The δ 1.029 shift, the protons of the C-18 methyl group, is coupled by ^2J to the carbon at δ 57.40 (C-14) and ^3J to carbons at δ 138.98 (C-13), 40.08 (C-8), and 30.93 (C-15). The carbons at δ 57.40 (C-14) and 40.08 (C-8) are coupled to protons at δ 0.911 (Me-30) and 1.029 (Me-18), respectively. These correlations establish the positions of C-18 and C-30 methyl groups. Furthermore, the presence of NOEs among Me-28/Me-19, Me-19/H-9, H-9/Me-18, Me-29/H-3, and H-3/H-5, as well as the absence of NOEs between Me-19/Me-30 and Me-18/Me-30, confirm the trans−syn−trans stereochemistry and the structure as protosta-13(17),24-dien-3β-ol, a product with $\triangle^{13(17),24}$ double bonds. Production of protosta-13(17),24-dien-3β-ol indicates that oxidosqualene enters the enzyme active site cavity with chair−boat−chair (C−B) conformation and initiates the cyclization/rearrangement cascade with four consecutive ring annulations to form the protosteryl C-20 cation, which is followed by a backbone rearrangement of H-17α→H-20α, a 1,2-hydride shift, to generate the protosteryl C-17 cation. Elimination of a proton at C-13 yields protosta-13(17),24-dien-3β-ol as the end product (Scheme 1). The ERG7^{F699T} mutant adds a new example of an oxidosqualene−lanosterol cyclase modified to make a truncated rearrangement product

Scheme 1. Product Profile Produced by *S. cerevisiae* TKW14 Expressing the ERG7^{F699X} Site-Directed Mutations



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(7) The in vivo TKW14[$_p$ERG7^{F699T}] product profile represent metabolic equilibrium of lanosterol and related triterpenes under physiological conditions, whereas the YTL4[$_p$ERG7^{F699T}] blocks metabolic flux of lanosterol into downstream sterol biosynthesis and allows quantification of mutant product profile from the in vivo accumulation.

accurately, and it compares favorably with proteins redesigned in other systems.[3a,4f] These results confirmed our working hypothesis and further validated the homology model.

It is remarkable that the replacement of the conserved Phe699 of ERG7 with Thr residue generated a mutant that biosynthesized the truncated rearrangement product, protosta-13(17),24-dien-3β-ol, with altered product specificity. The current results also validate the plasticity of the active-site residues. Specifically, a single atom difference in shape, volume, or polarity can govern the reaction product specificity. In addition, these experiments exemplify the power of homology modeling coupled with site-directed mutagenesis and product characterization to decipher structure−function relationships of the oxidosqualene cyclases in order to redesign a protein with tailored product specificity or diversity. Further studies should focus on characterizing the molecular interactions that influence the prefolded substrate conformation, the chair−boat bicyclic carbocation intermediate conformation, and the divergent evolution of new enzymatic functions.

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Supporting Information Available: Experimental details for the molecular cloning, incubation, separation of products, and spectroscopic data for protosta-13(17),24-dien-3β-ol. This material is available free of charge via the Internet at http://pubs.acs.org.

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