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Tryptophan 232 within Oxidosqualene–Lanosterol Cyclase from *Saccharomyces cerevisiae* Influences Rearrangement and Deprotonation but Not Cyclization Reactions

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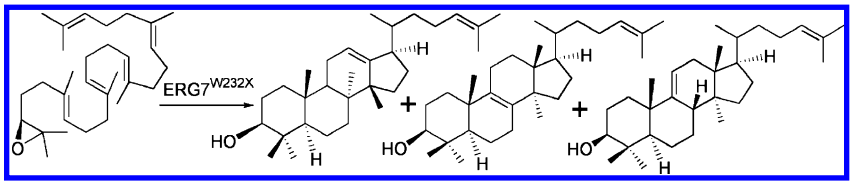
ABSTRACT



Oxidosqualene–lanosterol cyclases convert oxidosqualene to lanosterol in yeast and mammals. Site-saturated mutants' construction of *Saccharomyces cerevisiae* oxidosqualene–lanosterol cyclase, at Trp232 exchanges against proteinogenic amino acids, and product profiles are shown. All mutants, except Lys and Arg, produced protosta-12,24-dien-3β-ol, lanosterol, and parkeol. Overall, Trp232 plays a catalytic role in the influence of rearrangement process and determination of deprotonation position but does not involve intervention in the cyclization steps.

The enzymatic conversion of acyclic oxidosqualene into diverse polycyclic sterols and triterpenoids, which is catalyzed by oxidosqualene cyclases, represents one of the most remarkable one-step biotransformation processes found in nature.[1−3] Two biosynthetic routes, which have the following differences: folding of the substrate conformation, stabilization of the cationic intermediates and skeletal rearrangement, and deprotonation position, can be generally categorized and result in the production of diverse product profiles. This unique regio- and stereospecific polyolefinic cyclization/rearrangement reaction mechanism, and the molecular interactions between cationic intermediates and functional groups of catalytic amino acid residues of the oxidosqualene cyclase enzymes, has attracted many biological chemists to study structure−function relationships of the enzyme. Genetic selection and truncated product isolation, from oxidosqualene cyclase mutants, have led to identification of critical amino acid residues, which are involved in either

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product specificity change from cycloartenol to lanosterol or determining cyclization/rearrangement cascade outcome.[2,3] In addition, crystallographic analysis of both bacterial squalene−hopene cyclase (SHC) and human oxidosqualene−lanosterol cyclase (OSC) have provided a more in-depth analysis of structure−function relationships of (oxido)-squalene cyclases.[4,5] And yet, detailed molecular interactions between the substrate and enzyme for chair−boat 6−6 bicyclic B-ring and discrete 1,2-hydride and methyl group rearrangement, as well as deprotonation, are still far from being fully understood.

Rearrangement steps during the oxidosqualene cyclase-catalyzed reaction were suggested to be driven by either π-electron density gradient, at different active site regions, or only minimal assistance from the enzyme.[1c,4c,5] In one study, Corey used protostenediol benzoate as the substrate and nonenzymatically converted it to the corresponding dihydroparkeol benzoate, which suggested that minimal assistance is required of the enzyme during the rearrangement step.[5a] In the other study, Thoma et al. proposed, from the human OSC X-ray structure, that the rearrangement reactions were driven by the π-electron density gradient in the different active site region, and consequently terminated by the putative basic residue His232, when in the proximity of the termination site or the hydroxyl group of Tyr503, which is H-bonded to His232 and located in a better position to accept protons from the C-9 of the lanosteryl cation.[5b] We previously performed site-directed mutagenesis experiments on *Saccharomyces cerevisiae* oxidosqualene-lanosterol cyclase (ERG7) to elucidate putative amino acid residues that are involved in the cyclase-catalyzed deprotonation step. A putative base residue at the 510 position of ERG7, Tyr510 (corresponds to Tyr503 of human OSC), was mutated to alanine, and the resulting ERG7^{Y510A} mutant was subjected to genetic complementation and product characterization.[2a] The results obtained showed that the ERG7^{Y510A} mutant is viable and can generate multiple triterpene products, including an alternative deprotonation product parkeol. In addition, another putative base, His234 of ERG7 (corresponds to His232 of human OSC), was substituted with tyrosine and also resulted in a viable mutant and the isolation of multiple triterpenes, including incompletely cyclized achilleol A, truncated rearranged protosta-12,24-dien-3β-ol, lanosterol, and alternatively deprotonated product parkeol.[2b] Conversely, lanosterol was isolated as the sole product from an ERG7$^{H234Y/Y510A}$ double mutant. These findings support the hypothesis that the ERG7 His234:Tyr510 may function as follows: a catalytic base dyad in the control of final deprotonation, the plasticity of the dyad in response to other amino acid residues, and consequently in the change of product specificity.[5b] However, the possible involvement of other amino acid residues in cationic intermediate stabilization, during the rearrangement process or in the function as an alternative base for the final deprotonation step, could not be excluded.

To further elucidate possible involvement of other amino acid residues and their effect on the process of rearrangement and deprotonation, aromatic residues (with consensus of sequence among various oxidosqualene cyclases and spatially located between the C-20 and C-8/C-9 position of lanosterol) were subjected to genetic selection and product characterization. This was performed with the assumption that the higher the π-electron density gradient in different active site regions in the driving rearrangement process and their intrinsic reactivity to form cation−π interactions to carbocationic intermediates.[6] Among the various aromatic residues of ERG7 under investigation, Trp232 is of particular interest. The performed sequence analysis provided the result that Trp232 is highly conserved in oxidosqualene−lanosterol cyclase, oxidosqualene−cycloartenol synthase, and β-amyrin synthase, but instead substituted by either leucine or glycine, within lupeol synthase and squalene−hopene cyclase, respectively. In addition, mutagenesis studies of ERG7^{W232} were performed by Corey and co-workers, which suggested that Trp232Ala, Trp232Gly, and Trp232Phe were functional, but could be chemically inactivated by the substrate analogue, 20-oxa-2,3-oxidosqualene, which suggested a model for possible involvement of the residue in D-ring development.[7] However, product isolation and characterization were not carried out with this mutant enzyme to investigate its catalytic reaction mechanism effect and product profiles. A detailed investigation of the structural model suggested contribution of higher π-electron density near the C-12 position of lanosterol. Therefore, it is tempting to investigate the effect of substituting Trp232 of ERG7 with Leu, Gly, and Ala, as well as other amino acids, for possible involvement of the residue during rearrangement steps. We report, within this context, results of site-saturated mutation (W232X) and the product profile determination of each mutant. An identical product profile, which included truncated rearrangement product and lanosterol as well as parkeol, but with varying product proportions, was isolated from a strain that expressed ERG7^{W232X} as its only oxidosqualene-cyclase. These results suggested a catalytic role for Trp232 in ERG7 that can influence both rearrangement process and deprotonation position, but is not involved in the cyclization steps.

The ERG7^{W232X} site-saturated mutants were constructed and expressed in a yeast HEM1 ERG7 double-knockout mutant TKW14, which is only viable when supplied with exogenous ergosterol or complemented with ERG7 activity.[2a,c,8] The TKW14[pERG7^{W232X}] strain expressed ERG7^{W232X} as its only oxidosqualene cyclase. The genetic selection result demonstrated that TKW14[pERG7^{W232X}] site-saturated mutants allowed for ergosterol-independent growth, except for the ERG7^{W232K} and ERG7^{W232R} mutations. These results

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indicated that the Trp232 mutations were not detrimental to the essential activity of ERG7, except for the Lys and Arg substitutions. Next, TKW14[pERG7^{W232X}] mutant strains were cultured individually, nonsaponifiable lipids (NSL) extracts were prepared, and products were analyzed by GC−MS, as described previously.[2b] The NSL extracts of TKW14-[pERG7^{W232X}] mutants that produced triterpenoid products with a molecular mass of $m/z = 426$ were further acetylated and subjected to an $AgNO_3$-impregnated silica gel column for chromatographic purification and structure characterization with NMR (^{1}H, ^{13}C NMR, DEPT, HSQC, HMBC, and 2D-NOE).

Product profiles of the ERG7^{W232X} site-saturated mutations are summarized in Table 1. The $AgNO_3$-impregnated TLC

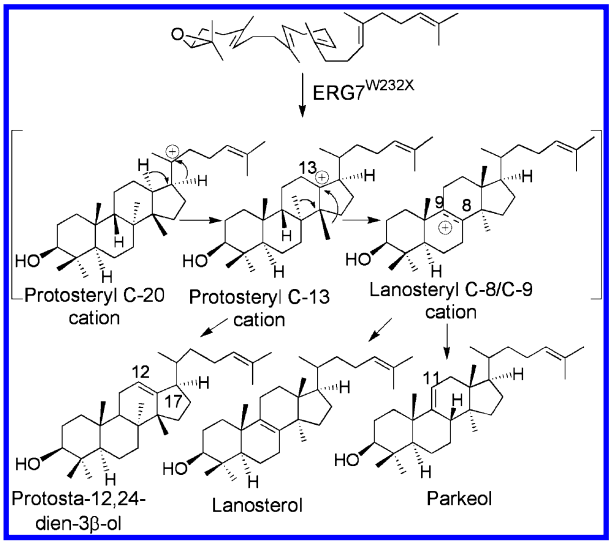
Table 1. The Product Profiles of *S. cerevisiae* TKW14 Expressing the ERG7^{W232X} Site-Saturated Mutants

amino acid substitution	protosta-12,24-dien-3β-ol	lanosterol	parkeol
native	0	100	0
W232G	8.4	74.4	17.2
W232A	18.1	47.1	34.8
W232V	19.3	34.0	46.7
W232I	23.5	33.0	43.5
W232L	14.5	27.8	57.7
W232D	7.9	84.8	7.3
W232E	14.0	49.2	36.8
W232S	10.7	59.0	30.3
W232T	14.3	61.7	24.0
W232N	10.7	59.5	29.8
W232Q	24.6	32.1	43.3
W232K	no new product		
W232R	pattern is the same as the negative control (TKW14)		
W232C	18.0	31.8	50.2
W232H	27.8	35.0	37.2
W232P	19.4	48.5	32.1
W232F	4.4	82.9	12.7
W232Y	4.2	94.2	1.6
W232M	10.9	40.6	48.5

and GC−MS results, from the NSL extracts, showed that no product with $m/z = 426$ could be observed for ERG7^{W232K} and ERG7^{W232R} mutations, consistent with ERG7 activity disruption. Conversely, three identical products, revealed on GC−MS with retention time and mass spectral characteristics consistent with that of protosta-12,24-dien-3β-ol (POL), lanosterol (LA), and parkeol (PK), respectively, but with different product ratios, could be identified from the NSL extracts of other ERG7^{W232X} mutants. Spectroscopic determination of each compound confirmed the above-mentioned structures, showing spectra that proved to be consistent with those of authentic compounds.[2b] Of all the ERG7^{W232X} mutants studied, the ERG7^{W232Y} mutant biosynthesized LA most accurately, whereas the W232L mutation favored PK and POL formation more than LA production. Interestingly, no monocyclic achilleol A or camelliol C was observed in the GC−MS analyses from any of the mutants.

How nature evolved the ERG7 gene in the eukaryote to form lanosterol, as the sole product, remains unknown. On the other hand, plant oxidosqualene-cyclases utilized the same substrate, but generated diverse product arrays with similar core structures. In ERG7^{W232X} mutants, oxidosqualene is cationic cyclized to a tetracyclic protosteroyl C-20 cation, without disruption at the monocyclic C-10 cation position. Then, a backbone rearrangement of H-17α → 20α, H-13α → 17α 1,2-hydride shifts to generate the C-13 cation, with elimination of H-12, yielding protosta-12,24-dien-3β-ol with the Δ^{12} double bond. Skeletal rearrangement of two methyl groups, originally derived from the C-14β and C-8α positions, and one hydride shift from H-9β to H-8β, will generate the lanosteryl C-8/C-9 cation, which undergoes deprotonation at C-8 and C-11 to form lanosterol and parkeol, respectively (Scheme 1).

Scheme 1. Product Profile Produced by *S. cerevisiae* TKW14 Expressing the ERG7^{W232X} Site-Saturated Mutations



With only a limited understanding of variability in metabolic flux ratios of POL, LA, and PK within the in vivo system, the product profiles reported herein provide some qualitative insights into the ERG7-catalyzed cyclization/rearrangement mechanism. We provided a plausible explanation as to how the substitution of Trp232, with other amino acid residues, impacts the difference in stabilization of rearrangement process and regulates deprotonation postions by use of structure models derived from human OSC crystal structure and complexed with lanosterol and protosteryl C-13 cation structure, respectively.[2a,b,4c,9] The structure of protosteryl C-13 cation was constructed and optimized at the B3LYP/6-31G* level of theory using Gaussian 03 software, reasoning the difference in spatial loactions of the methyl groups at C-8 and C-14 positions, between POL and C-8/C-9 carbocation products, LA and PK. Similar structure integrity was observed between lanosterol-complexed and

(9) Frisch, M. J.; et al. *Gaussian 03*, revision C.02; Gaussian, Inc.: Wallingford, CT, 2004.

protosteryl C-13 cation-complexed homology models, in both the distribution of secondary structure and 3-D profile (Figure 1).

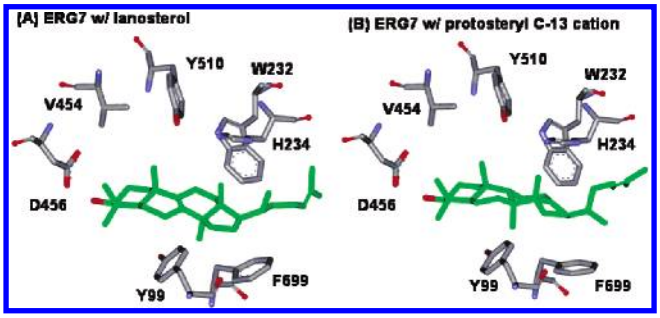


Figure 1. ERG7 residues interacting with C-11/C-12 of lanosterol and prtosteryl C-13 cation, respectively, in the homology model structures: (A) ERG7-complexed with lanosterol and (B) ERG7-complexed with prtosteryl C-13 cation.

As previously established, His234 and the Tyr510 residues of *S. cerevisiae* ERG7 are strictly spatial, as conserved with corresponding residues, within the human OSC, and there is consistency between the mutant homology model and the mutant product profile distribution.[2] Careful examination of the homology model suggested that Trp232 is positioned at the top half of the side wall of the active site cavity and proximal to C-11, C-12, and C-13 orientations of the products. In addition, the Trp232 is also spatially close to His234 and Tyr510, which occupy part of the ceiling of the active site and are H-bonded to each other. Homology simulation of the Trp232 position with other residues showed that changes at this position reduce the π-electron density nearby and affect the interactions of active site residues, especially the His234:Tyr510 catalytic base dyad H-bonding network, as well as shift the relative distance between His234 and C-11/C-12 position of LA. For example, substitution of Trp232 with nonpolar hydrophobic side chains such as Gly, Ala, Val, and Ile enlarged the cavity of the active site. This, in turn, impaired the His234:Tyr510 catalytic base dyad H-bonding network and slightly pulled His234 away from its original position and toward the C-11 and C-12 position to form PK and POL, respectively. Consistent with this observation is the production of more PK + POL than LA, as the side chain length was increased from H to the isobutyl group (Table 1). Alternatively, the change from Trp232, to Tyr or Phe, retained the His234:Tyr510 H-bonding network and only slightly reduced active site steric hindrance and the π-electron density. This minor reduction of the ligand−enzyme interaction resulted in LA production as the major product, but with production of minor amounts of PK and POL.

It has been suggested that a higher π-electron density, near the C-8/C-9 positions of LA with seven aromatic residues, might be responsible for the equilibrium shift toward the C-8/C-9 cation and, subsequently, for the lanosterol formation.[5b] In ERG7^{W232X} mutants, partial deprotonation, for POL formation, occurred prior to the shift of two methyl groups (Me-14β → Me-13β and Me-8α → Me-14α) for generating lanosteryl C-8/C-9 cation and subsequent formation of LA and PK. Quantum mechanical calculation showed that formation of LA and PK are thermodynamically favored by about 21.82 and 21.36 kcal/mol, respectively, relative to POL (see the Supporting Information).[9] In addition, direct deprotonation of protosteryl C-13 cation to form protosta-12,24-dien-3β-ol were thermodynamically unfavorable. Perhaps the substitution of Trp232, with other amino acid residues, resulted in the loss of cation−π interactions, which would destabilize the carbocation and facilitate deprotonation. Alternatively, the mutations at Trp232 would lead to stabilization or destabilization of various carbocation intermediates relative to the wild-type enzyme, which subsequently affects the relative rates of deprotonation and rearrangement. Further studies should focus on elucidation of precise molecular interactions on relative rates of deprotonation and rearrangement.

These performed experiments demonstrated plasticity of the ERG7 mutant enzyme and that mutations, at Trp232 position, change active site structure. This led to stabilization or destabilization of various carbocation intermediates, relative to the wild-type enzyme, and impaired catalytic fidelity of the enzyme, resulting in generation of multiple triterpene regioisomers. However, exact steric or electrostatic contributions of the mutations on the trajectories of product proportions, and rates for different products formation, remain unclear, awaiting creation of high-resolution crystal structures of mutated proteins, as well as kinetic analysis, which is currently under investigation.

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Supporting Information Available: General procedures and spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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