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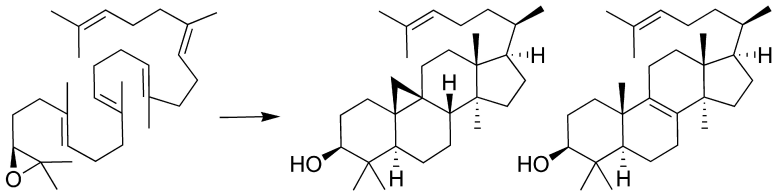

*Ath*CAS1	99%	0%
*Ath*CAS1H477N I481V	0%	99%

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Enzyme Redesign: Two Mutations Cooperate to Convert Cycloartenol Synthase into an Accurate Lanosterol Synthase

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Nature has used random mutagenesis and selection to generate an enormous diversity of enzymes. Protein scientists have recently begun to make progress in engineering enzymes with broadened substrate, reaction, and product specificity.[1] It has proven to be dramatically more difficult to redesign enzymes to have stringent specificity. Described herein are experiments in which homology modeling was combined with site-directed mutagenesis to redesign cycloartenol synthase to make lanosterol with 99% accuracy.

Oxidosqualene cyclases convert acyclic (*S*)-2,3-oxidosqualene (**1**) to over 100 cyclic triterpenes ($C_{30}H_{50}O$ and $C_{30}H_{52}O_2$).[2] The reaction is initiated by epoxide protonation, followed by cation−olefin cyclization and cation rearrangement, and terminated either by deprotonation or by water addition. Cycloartenol synthase is a member of this enzyme family that generates cycloartenol (**2**), the sterol precursor in plants and some protists. Lanosterol synthase is a paralog that forms lanosterol (**3**), the sterol precursor in animals and fungi. These enzymes are only moderately related (∼40% identical) but promote mechanistically similar reactions. Both enzymes cyclize oxidosqualene to the protosteryl cation (**4**) and promote hydride and methyl shifts to form the C-8 cation (**5**) and then diverge by abstracting a different proton (Scheme 1). Cycloartenol synthase promotes an additional hydride shift and terminates the reaction with cyclopropyl ring formation and deprotonation from C-19, whereas lanosterol synthase deprotonates from C-9. We elucidated how the known catalytically important residues impact the difference in deprotonation using a structural model derived from the *Alicyclobacillus acidocaldarius* squalene−hopene cyclase (*Aac*SHC) crystal structure.[3] The orientation of active-site side chains was refined using the recently reported crystal structure of the human lanosterol synthase (see Supporting Information).[4] We established previously that Tyr410, His477, and Ile481 are strictly conserved, catalytically important residues in *Arabidopsis thaliana* cycloartenol synthase[5] (*Ath*CAS1). These residues synergize to promote cycloartenol biosynthesis, and mutations at these positions allow lanosterol formation. Ile481 is conserved in all cycloartenol synthases, whereas Val is present in lanosterol synthases. The Ile481 γ-methyl promotes accurate cycloartenol formation by preventing rotation of the intermediate through steric interactions with C-2 and the two axial methyl groups of the A-ring. Removing the γ-methyl group with an Ile481Val mutation results in 25% lanosterol in addition to cycloartenol and parkeol (**6**) (Table 1).[6] Tyr410 hydrogen-bonds with His257 to form part of the ceiling of the active site.[3d] Both Tyr410 and His257 are close to C-19, and one of these could be the base that deprotonates to form cycloartenol. Alternatively, their influence on deprotonation could result from participation in the H-bonding network. Tyr410 is present in all cycloartenol synthases, but animal and fungal lanosterol synthases maintain Thr at the corresponding position. The *Ath*CAS1 Tyr410Thr mutant forms 65% lanosterol along with 9β-lanosta-7,24-dien-3β-ol (**7**) and parkeol.[7] Removing the aromatic ring of Tyr410 decreases steric bulk above the intermediate. Because the hydroxyl in Thr is closer to the α-carbon than in Tyr, the polar groups of Tyr410Thr, Tyr532, and His257 are repositioned in the Tyr410Thr mutant. This combination of steric and electronic changes abolishes cycloartenol biosynthesis and allows deprotonation of the C-8/C-9 lanosteryl cation to form products **3**, **6**, and **7**. His477 is not in the active site, but is a second-sphere residue that affects the product profile through interactions with the side chain of Tyr410.[3d] His477 is strictly conserved in the known cycloartenol synthases, whereas lanosterol synthases maintain either Gln or Cys. The *Ath*CAS1 His477Gln mutant is a parkeol synthase (73%) that also forms lanosterol (22%).[8] Although known lanosterol synthases lack Asn at this position, an *Ath*CAS1 His477Asn mutant biosynthesizes 88% lanosterol and 12% parkeol.[8] The structural model suggests that these His477 mutations alter steric and electronic surfaces of the active site by offering a smaller interaction partner to Tyr410, which is consequently withdrawn from the active site. The Tyr410Thr mutation facilitates lanosterol production by decreasing active-site bulk and relocating potential proton acceptors, and the His477 mutants achieve a similar result by moving Tyr410 away from the intermediate cation.

Scheme 1. Oxidosqualene Cyclases Convert Oxidosqualene to Diverse Cyclic Structures

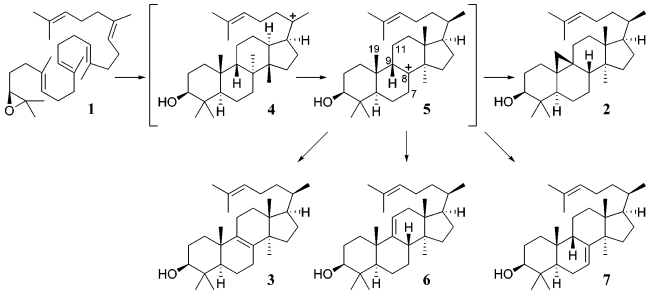

Table 1. % Yields of Cycloartenol (**2**), Lanosterol (**3**), Parkeol (**6**), and 9β-Δ7-Lanosterol (**7**) in Cycloartenol Synthase Mutants

mutant	2	3	6	7
native	99	0	1	0
H477N	0	88	12	0
H477Q	0	22	73	5
I481V	54	25	21	0
Y410T	0	65	2	33
Y410T/I481V	0	78	<1	22
Y410T/H477N/I481V	0	78	0	22
Y410T/H477Q/I481V	0	78	0	22
H477N/I481V	0	99	1	0
H477Q/I481V	0	94	6	0

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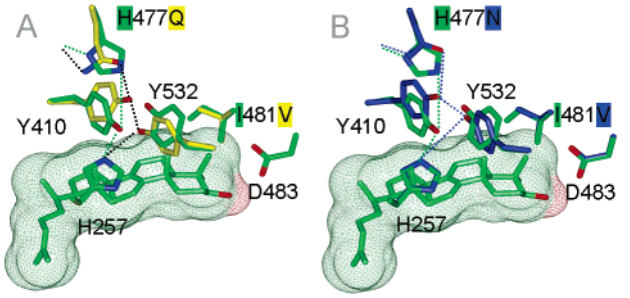

Figure 1. Superposition of wild-type *Ath*CAS1 (green) with (A) *Ath*CAS1 H477Q I481V (yellow) and (B) *Ath*CAS1 H477N I481V (blue). The lanosteryl cation is shown with a transparent Connolly surface. The hydrogen-bonding pattern is indicated by dotted lines.

Careful examination of the homology model suggested that the His477Gln and His477Asn mutations should synergize with the Ile481Val mutation to more accurately biosynthesize lanosterol. The *Ath*CAS1 His477Gln mutant has the polar functionality moved toward C-11 and consequently biosynthesizes more parkeol than lanosterol. *Ath*CAS1 His477Asn forms lanosterol by positioning the base near C-9/C-8, but close enough to C-11 to form some parkeol. *Ath*CAS1 Ile481Val allows some lanosterol biosynthesis by introducing a smaller side chain, which enlarges the active-site cavity, permitting rotation of the intermediate cation. Computer models show that both the *Ath*CAS1 His477Gln Ile481Val and the *Ath*CAS1 His477Asn Ile481Val double mutants relocate polarity to a position more favorable for lanosterol formation. The decreased sterics allow the intermediate to rotate, moving C-9/C-8 toward the base. Because *Ath*CAS1 His477Gln and *Ath*CAS1 His477Asn form 73 and 12% parkeol, respectively, the reduction in parkeol biosynthesis in *Ath*CAS1 His477Gln Ile481Val was expected to be less than that in *Ath*CAS1 His477Asn Ile481Val.

The *Ath*CAS1 His477Asn Ile481Val and the *Ath*CAS1 His477Gln Ile481Val double mutants were expressed in the yeast lanosterol synthase mutant SMY8.[9] Both mutant enzymes genetically complemented the mutation, whereas *Ath*CAS1 did not. Expression in the yeast squalene epoxidase/lanosterol synthase double mutant RXY6[10] provided cyclase free of in vivo products, and in vitro assay with racemic oxidosqualene generated a triterpene alcohol fraction. After purification by silica gel chromatography and derivatization as the trimethylsilyl (TMS) ethers, GC−FID and GC−MS of the *Ath*CAS1 His477Asn Ile481Val mutant showed a major peak (99%) with retention time and MS identical to that of a TMS−lanosterol standard. The GC−MS spectra also revealed the presence of TMS−parkeol (1%). The structural assignments were confirmed by 500 MHz ^{1}H NMR analysis. Similar analyses on the *Ath*CAS1 His477Gln Ile481Val mutant products revealed the same products in a ratio of 94 and 6%. If any additional byproducts were present, they were at levels <1%. The GC−FID ratios are in close agreement with the ratios obtained by NMR quantitation. These rationally designed cycloartenol synthase derivatives are the best known examples of terpene synthases modified to make different products accurately, and they compare favorably with remodeled proteins in other systems. The experimental results for both double mutants confirmed our working hypothesis and further validate the homology model. Activity assays indicated that the *Ath*CAS1 His477Asn Ile481Val mutant has about half the activity of wild-type *Ath*CAS1.[11]

The synergistic behavior of the His477Asn/Gln and Ile481Val mutations resembles that seen with the Tyr410Thr and Ile481Val mutations; the *Ath*CAS1 Tyr410Thr Ile481Val double mutant produces lanosterol more accurately (78%) than the parent mutants.[3d,7] Neither mutation depends on direct interaction with the other or preempts the effects of the other. In contrast, the His477Asn and His477Gln mutations lack influence when both Ile481Val and Tyr410Thr are present (Table 1).[3d] These three mutations are not synergistic because, when Tyr410 is mutated to Thr, His477Asn/Gln cannot interact with the smaller and more distant Thr side chain and, therefore, cannot influence catalysis.

The *Ath*CAS1 His477Asn Ile481Val double mutant (with native Tyr410) is the most accurate example of an enzyme mutated to biosynthesize lanosterol, and the *Ath*CAS1 His477Gln Ile481Val double mutant is nearly as accurate. Neither enzyme has the Tyr410Thr Ile481Val maintained by animal and fungal lanosterol synthases. Although no known native enzyme uses the motifs of the more accurate *Ath*CAS1 His477Asn Ile481Val double mutant, the *Ath*CAS1 His477Gln Ile481Val double mutant has the residues of the phylogenetically distinct trypanosomal lanosterol synthases.[12]

The experiments described here show how mutagenesis coupled with a high-quality model allows the design of an efficient and highly accurate lanosterol synthase. An improved understanding of structure and catalytic mechanisms should facilitate future engineering of enzymes with tailored catalytic activities.[13]

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

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