Conversion of a Plant Oxidosqualene-Cycloartenol Synthase to an Oxidosqualene-Lanosterol Cyclase by Random Mutagenesis†

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ABSTRACT: A random mutagenesis/in vivo selection approach was applied to generate and identify mutations that alter the product specificity of oxidosqualene-cycloartenol synthase (CAS) from *Arabidopsis thaliana*. This work complements previous studies of triterpene cyclase enzymes and was undertaken to provide knowledge of the frequency and locations at which point mutations can alter cyclase product specificity. Random mutations were introduced by treatment with hydroxylamine or passage through a mutator strain of bacteria. Libraries of mutated plasmids carrying the *cas1* gene were transformed into a cyclase-deficient strain of *Saccharomyces cerevisiae* (CBY57) bearing a complementing plasmid (pZS11) carrying an *Erg7* gene that encodes wild-type yeast oxidosqualene-lanosterol cyclase and a URA3 marker that could be counterselected by growth in media containing 5-fluoroorotic acid (5-FOA). This allowed use of a plasmid shuffle to select for *cas1* mutants that could substitute for ERG7 activity. Five of ∼73000 transformants were observed to grow in media containing 5-FOA but lacking ergosterol. pTKP5-derived plasmids isolated from these transformants were sequenced, revealing five distinct and unique point mutations: Tyr410Cys, Ala469Val, His477Tyr, Ile481Thr, and Tyr532His. Analysis of the nonsaponifiable lipids from CBY57 cells expressing these mutants suggests that the Tyr410Cys and His477Tyr mutants produce lanosterol as the dominant product, whereas the Ala469Val, Ile481Thr, and Tyr532His mutants produce a mixture of lanosterol and achilleol A, a product of monocyclization. Sequence and structural homology modeling of CAS indicate that the observed product specificity-altering mutations occur both within (Tyr410Cys, Ile481Thr, and Tyr532His) and outside of (Ala469Val and His477Tyr) the cyclase active site.

The family of triterpene cyclase enzymes catalyze highly diverse and complex cyclization/rearrangement reactions (*1*−*3*). In fungi and mammals, oxidosqualene-lanosterol cyclase (ERG7,[1] EC 5.4.99.7) mediates the conversion of (3*S*)-2,3-oxidosqualene into lanosterol, whereas this same substrate is elaborated to cycloartenol through the agency of oxidosqualene-cycloartenol synthase (CAS, EC 5.4.99.8) in algae and photosynthetic plants. In prokaryotes, squalene-hopene cyclase (SHC) catalyzes the conversion of squalene to pentacyclic triterpenoids (Figure 1).

Elegant biogenetic and bioorganic investigations, including studies of the structures of novel products formed from designed alternate substrates, have provided a detailed model for cyclase-mediated substrate transformation (*4*−*11*). In this model, enzymes initiate cyclization cascades by protonating the terminal double bond of squalene or the oxirane moiety of oxidosqualene. The enzymes then promote cyclization cascades leading, in the case of ERG7 and CAS, to an intermediate protosterol cation which undergoes a series of hydride and methyl shifts. Lanosterol results from C-9 deprotonation of a C-8 cation, while cycloartenol forms after a further hydride migration and deprotonation of the C-10 methyl group.

During the past decade, direct studies of triterpene cyclase enzymes have been facilitated by the cloning of dozens of cyclase genes (*1*−*3* and references therein), while interpretation of results has been greatly aided by the crystallization and structural characterization of the SHC from *Alicyclobacillus acidocaldarius* (*12*, *13*). A significant thrust of mutagenesis experiments has been the analysis of factors that determine product specificity (*14*−*26*), perhaps the single most remarkable feature of these enzymes. Of particular relevance to the present study, Matsuda and co-workers have reported the use of site-directed mutagenesis and directed evolution in identifying and probing in detail active site residues of ERG7 and CAS enzymes that have an impact on product specificity in oxidosqualene cyclase enzymes (*19*−*26*). We now report a random mutagenesis/in vivo selection approach to identifying changes in the oxidosqualene-cycloartenol synthase from *Arabidopsis thaliana* that impart this enzyme with oxidosqualene-lanosterol cy-

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[1] Abbreviations: ERG7, oxidosqualene-lanosterol cyclase; CAS, oxidosqualene-cycloartenol synthase; SHC, squalene-hopene cyclase; PCR, polymerase chain reaction.

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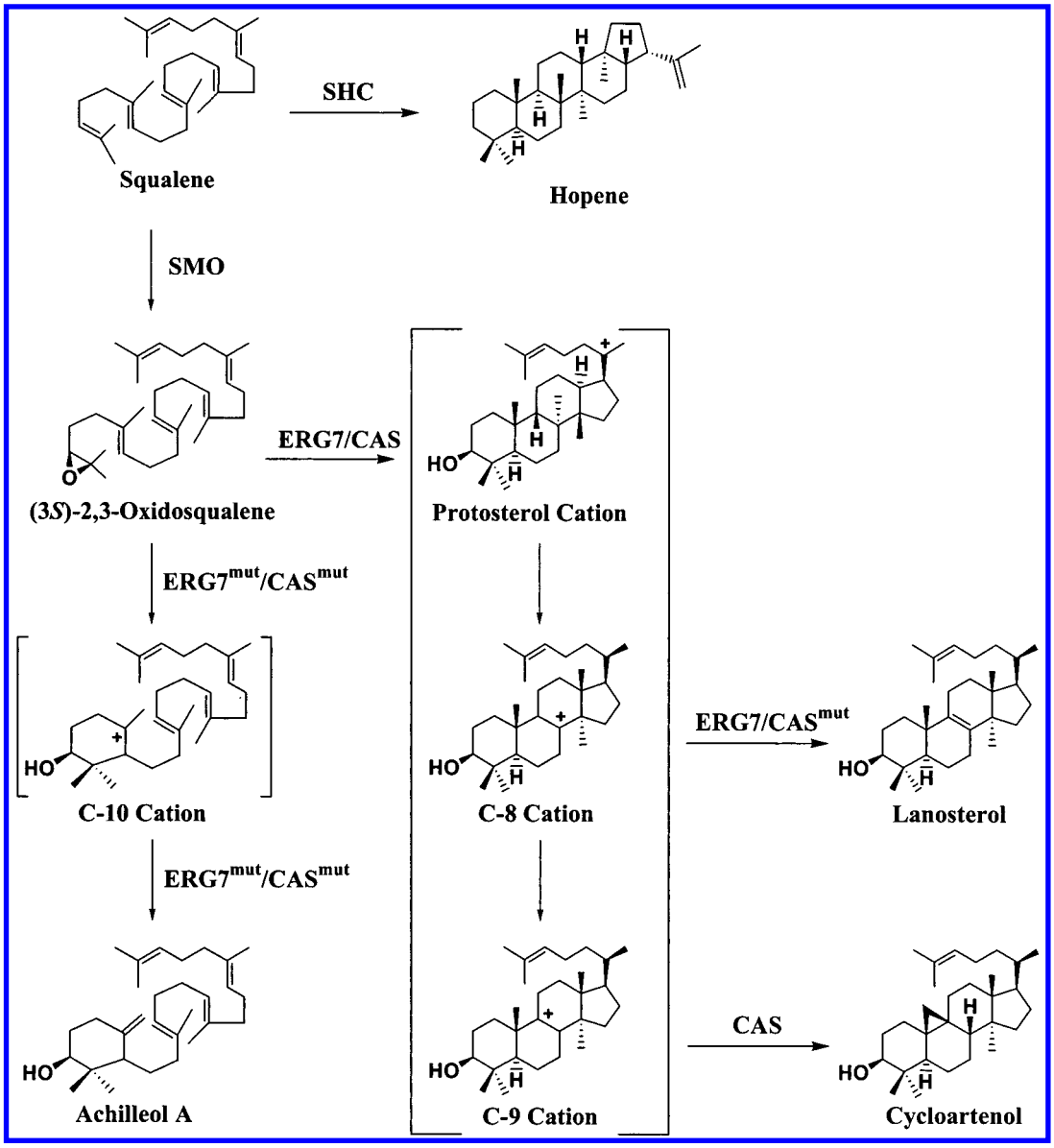

FIGURE 1: Triterpene cyclization reactions. Squalene-hopene cyclase (SHC) catalyzes the conversion of squalene to hopene. Squalene monooxygenase (SMO) catalyzes the conversion of squalene to (3*S*)-2,3-oxidosqualene. Oxidosqualene-lanosterol cyclase (ERG7) and oxiodsqualene-cycloartenol synthase (CAS) catalyze the cyclization of (*3S*)-2,3-oxidosqualene to lanosterol and cycloartenol, respectively, via a common tetracyclic protosterol cation intermediate. The protosterol cation is then rearranged and deprotonated to produce either lanosterol (formally via a C-8 cation) or cycloartenol (formally via a C-9 cation). Mutated forms of ERG7 and CAS (ERG7^{mut} and CAS^{mut}) may display modified product specificities, resulting in the formation of alternate tetracycles or products of aborted cyclization such as achilleol A.

clase activity. This has allowed us to identify five unique product specificity-altering point mutations and to estimate the frequency with which such mutations arise. Three of the mutations that have been isolated occur at positions not known previously to have an impact on product selectivity in the oxidosqualene cyclase family, and two of these appear to lie outside of the enzyme active site.

MATERIALS AND METHODS

Strains and Plasmids. Except where stated otherwise, bacterial growth, agar plate preparation, recombinant DNA purification and identification, and agarose gel electrophoresis were performed according to standard procedures (*27*). Phagemid pBluescript II SK(+), *Escherichia coli* XL1-Blue, and the *Epicurian coli* mutator strain XL1-Red deficient in DNA repair were purchased from Stratagene. Plasmid vector pRS313 was kindly provided by D. Botstein (Stanford University). Plasmid DNA was extracted from yeast by standard methods, and transformed into XL1-Blue for large-scale preparation and further analysis (*28*, *29*). The haploid yeast strain CBY57([pZS11]) (ERG7Δ::LEU2 *ade2*-101 *lys2*-801 *his3*-Δ200 *leu2*-Δ1 *trp1*-Δ63 *ura3*-52 [pZS11]) was derived from the diploid strain CBY1 (MATa/α ERG7Δ::LEU2 *ade2*-101 *his3*-Δ200 *lys2*-801 *trp1*-Δ63 *ura3*-52) (*30*). CBY1 was grown on YPD medium and transformed by electroporation with plasmid pZS11 (*29*, *30*). Transformants were selected on SDC medium without uracil, sporulated in YPA/SPM, and dissected. Dissected spores were selected on SDC media lacking both leucine and uracil.

Construction of the Hybrid Erg7/cas1 Plasmid. To facilitate expression of the active CAS protein in yeast, a hybrid ERG7/CAS expression plasmid (pTKP5) was constructed in

which the promoter sequence from the *Erg7* gene was fused to the coding sequence from the *cas1* gene. A *Not*I−*Xho*I restriction fragment of the *cas1* gene [prepared by the standard PCR methodology using 5′-d(ATAAGAATGCGGCCGCGGATCCCATATGTGGAAACTGAAGATCGCGGAAGG)-3′ and 5′-d(GTAAAACGACGGCCAGT)-3′ as primers and PTKCAS-SK1 as a template] was isolated and ligated with the yeast−*E. coli* shuttle vector pRS313 previously digested with the same enzymes, to construct plasmid pTKP1. A 0.3 kbp DNA fragment of the *Erg7* promoter region [prepared by the standard PCR methodology using 5′-d(ATAAGAATGCGGCCGCGAGCTACGTCAGGGCCCCTA)-3′ and 5′-d(TCTAGAATTCCATATGTTTTGTACTTTCTTTGTG)-3′ as primers and pZS11 as a template] was isolated and ligated into the *Not*I−*Nde*I sites of pTKP1 to construct the hybrid expression plasmid pTKP5.

Random Mutagenesis, Transformation, and Plasmid Shuffle Selection. Plasmid pTKP5 was subjected to chemical mutagenesis or to the *E. coli* mutator strain XL1-Red to generate libraries of randomly mutagenized plasmids. For chemical mutagenesis, 10 μg of pTKP5 DNA was incubated with 500 μL of a hydroxylamine solution [containing 1 M hydroxylamine, 50 mM sodium pyrophosphate (pH 7.0), 100 mM sodium chloride, and 2 mM EDTA] at 75 °C for 30 min (*31*). After the mixture had been cooled on ice, DNA was separated by gel filtration (Sephadex G-25). For mutagenesis in the mutator strain, pTKP5 was transformed into XL1-Red, cultured for approximately 45 generations in LB broth, and isolated according to standard procedures (*27*, *32*, *33*).

Mutagenized pTKP5 DNA (pTKP5*) was introduced into yeast strain CBY57([pZS11]) by electroporation. Yeast were plated and grown on SD+Ade+Lys+Trp+1 M sorbitol medium to select for the presence of both pZS11 and pTKP5-derived plasmids. The resulting libraries of CBY57([pZS11])([pTKP5*]) transformants were grown overnight in 10 mL of SD+Ade+Lys+Trp+Ura medium at 30 °C. Aliquots (200 μL) from each culture were plated on SD+Ade+Lys+Trp+Ura and SD+Ade+Lys+Trp+Ura+1 mg/mL 5-fluoroorotic acid (5-FOA) medium and grown for 2−3 days at 30 °C. Colonies that grew on 5-FOA-containing plates were grown individually in SD+Ade+Lys+Trp+Ura liquid medium. The presence of pTKP5-derived plasmids, but not pZS11, was verified using yeast minipreps and restriction endonuclease digestion.

DNA Sequencing of Mutant cas Genes. DNA sequencing of pTKP5-derived plasmids isolated through the plasmid shuffle procedure was carried out by the dideoxy chain-termination method using ABI PRISM BigDye Terminator Cycle Sequencing Reaction kits on an Applied Biosystems 373A DNA sequencer (*34*).

Cyclase Activity Assay. Cyclase activity in cell-free yeast lysates was monitored using a radio-TLC assay with [^{3}H]-2,3-oxidosqualene as described elsewhere (*29*). Radioisotope distribution on the TLC plates was obtained either by scintillation counting or by direct scanning with a Bioscan AR-2000 instrument. The degree of conversion was determined by scintillation counting of the silica gel containing oxidosqualene and sterols from each lane of the TLC plate, using a Packard Tri-Carb 2900TR scintillation counter.

Lipid Extraction, Column Chromatography, and Identification of an Alternative Cyclization Product. A 4 L culture of the CBY57([pTKRMY68]) transformant was grown in SD+Ade+Lys+Trp+Ura medium at 30 °C with shaking (220 rpm) for 3 days. The cells were harvested by centrifugation, washed, and saponified by refluxing them in 250 mL of a 15% KOH/50% EtOH mixture for 3 h (*35*). The hydrolysate was extracted twice with 500 mL of petroleum ether, and the combined extracts were concentrated using a rotary evaporator. The extract was fractionated by silica gel column chromatography using a 19:1 hexane/ethyl acetate mixture. Further fractionation was performed using high-performance liquid chromatography (HPLC) using an Intersil 7 ODS-3 column (7.6 mm inside diameter × 250 mm) with acetonitrile as the eluent (flow rate of 3.0 mL/min, detection at 202 nm) at room temperature. Under these conditions, a non-lanosterol product was observed with a retention time of 22.5 min. This material was identical to achilleol A according to TLC (R_f = 0.43, silica gel, 5:1 hexane/ethyl acetate mixture), ^{1}H and ^{13}C NMR, IR, and MS analyses, yielding spectra consistent with data available in the literature (*22*, *26*, *36*): ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.696 (s, 3H), 1.011 (s, 3H), 1.237 (s, 2H), 1.535−1.661 (m, 12H), 1.741−1.846 (m, 3H), 1.954 (t, J = 7.3 Hz, 2H), 1.98−2.084 (m, 14H), 2.298 (dt, J = 13.1 and 4.7 Hz, 1H), 3.380−3.391 (dd, J = 9.9 and 4.2 Hz, 1H), 4.587 (s, 1H), 4.854 (s, 1H), 5.084−5.129 (m, 4H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 15.98, 16.01, 16.04, 17.66, 23.71, 25.67, 25.87, 26.67, 26.76, 28.24, 28.29, 29.68, 32.22, 33.09, 38.61, 39.71, 39.75, 40.53, 50.90, 77.30, 108.35, 124.26, 124.29, 124.36, 124.41, 131.20, 134.88, 135.10, 135.41, 147.24; MS m/z 426 ($[M]^+$), 408, 357, 339, 271, 203, 175, 149, 135, 121, 95, 81.

Molecular Modeling of Oxidosqualene-Cycloartenol Synthase. The GeneMine program developed by the Molecular Application Group was used to generate plausible conformations for CAS based on known X-ray structures, particularly for SHC from *A. acidocaldarius* (*12*, *13*, *37*, *38*). The amino acid sequence of CAS was first aligned with that of SHC, and then used to search for matching segments from the database on the basis of both the amino acid sequence and the conformational similarities. The resulting structure was energy minimized using the program ENCAD, which employs a standard interatomic potential energy function consisting of bond stretching, angle bending, torsional, nonbonded, and electrostatic interactions (*39*).

RESULTS

Construction of Randomly Mutagenized Libraries of the cas1 Gene. Two independent approaches were taken to generate random mutations in the *cas1* gene from *Ar. thaliana*. Both approaches employed yeast−*E. coli* shuttle vector pTKP5 in which the promoter sequence from the yeast *Erg7* gene was fused to the coding sequence from the *cas1* gene to facilitate expression in *Saccharomyces cerevisiae*. In one approach, pTKP5 was subjected to chemical mutagenesis by hydroxylamine which promotes C to T and G to A transition mutations (*31*). In the second approach, pTKP5 was passed through approximately 45 generations of the *Ep. coli* XL1-Red mutator strain deficient in DNA repair (*32*, *33*).

Screening for cas Mutants That Are Able To Complement Yeast Mutants Deficient in osc Using a Plasmid Shuffle. The two libraries of mutated pTKP5 DNA were isolated and transformed by electroporation into *S. cerevisiae* strain

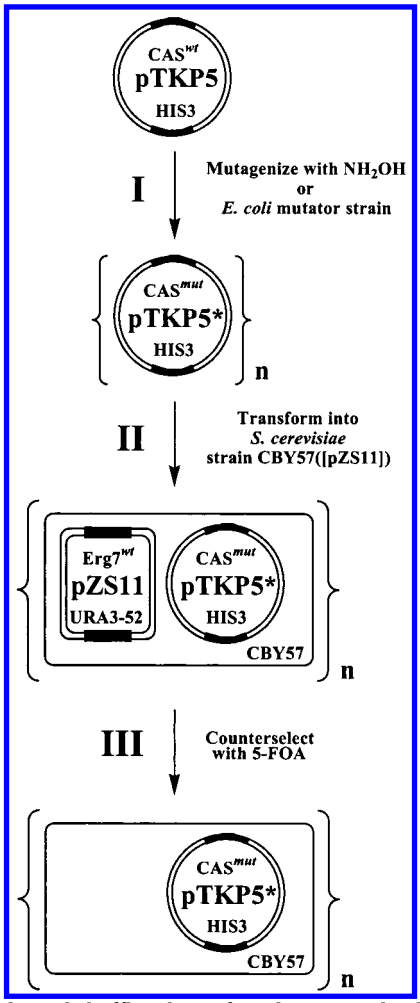

FIGURE 2: Plasmid-shuffle scheme for selecting randomly generated CAS mutants for the ability to catalyze the formation of lanosterol. In step I, random mutations are introduced into the wild-type *cas* gene (CAS^{wt}) either by treating recombinant plasmid pTKP5 with hydroxylamine or by passing it through an *E. coli* mutator strain. In step II, libraries of randomly mutated *cas* genes (CAS^{mut}), carried on mutant plasmids pTKP5*, are transformed into *S. cerevisiae* strain CBY57 harboring plasmid pZS11 and grown on media that select for the presence of both plasmids. PZS11 carries a copy of the wild-type *Erg7* gene from *S. cerevisiae* which complements the insertionally inactivated chromosomal *Erg7* gene. PZS11 also carries the selectable and counterselectable URA3-52 marker. In step III, CBY57([pZS11])([pTKP5*]) transformants are grown on media containing 5-FOA but lacking ergosterol, which selects against cells carrying pZS11 and selects for cells carrying pTKP5* plasmids capable of complementing the cyclase deficiency of CBY57.

CBY57([pZS11]). This strain (ERG7Δ::LEU2 *ade2*-101 *lys*2-801 *his3*-Δ200 *leu2*-Δ1 *trp1*-Δ63 *ura3*-52) bears an insertionally inactivated *Erg7* gene which is complemented by the wild-type gene carried on plasmid pZS11 (*29*, *30*). Plasmid pZS11 also bears the URA3 marker that can be counterselected by 5-FOA. This allowed us to use a plasmid shuffle strategy to screen for *cas1* mutants that could complement the oxidosqualene-lanosterol cyclase deficiency of CBY57 (Figure 2) (*40*).

Yeast cells were first selected for the presence of both pZS11 and pTKP5-derived plasmids. Colonies were then replicated using nitrocellulose membranes onto plates containing 1 mg/mL 5-FOA but lacking ergosterol to select against pZS11 and to select for *cas1* mutants capable of supporting growth. One of ∼18300 replicated colonies bearing pTKP5* modified by chemical mutagenesis and four of ∼55000 replicated colonies bearing pTKP5* passed through the *Ep. coli* mutator strain were observed to grow under these conditions.

Plasmid DNA isolated from these five colonies was shown by restriction analysis to be pTKP5-derived and not pZS11. Sequencing of the plasmids revealed that each carried a single, unique nucleotide substitution within the coding region of the *cas1* gene which corresponds to a single, unique amino acid substitution within the CAS enzyme. The isolated plasmids, their observed nucleotide changes, and their presumed amino acid changes are as follows: (1) pTKRMY21, C1429T, and His477Tyr, (2) pTKRMY57, A1229G, and Tyr410Cys, (3) pTKRMY61, C1406T, and Ala469Val, (4) pTKRMY64, T1443C, and Ile481Thr, and (5) pTKRMY68, T1594C, and Tyr532His.

Isolation and Characterization of Cyclized Triterpene Products. The selected CBY57([pTKP5*]) transformants were grown in liquid media and harvested, and their nonsaponifiable lipids (NSLs) were extracted. Thin-layer chromatography (TLC) indicated the presence of both lanosterol and ergosterol in all of the transformed cells. Lanosterol and ergosterol were the only cyclized triterpenes identified in cells expressing the Tyr410Cys and His477Tyr mutants. In contrast, cells expressing the Ala469Val, Ile481Thr, and Tyr532His mutants accumulated significant quantities of a third NSL. This compound was isolated at preparative scale from a culture of CBY57([pTKRMY68]) and characterized by HPLC, GC−MS, and NMR. These results served to identify the product as the monocyclic triterpene achilleol A by comparison with published data (*22*, *26*, *36*).

Sequence and Structural Homology Modeling of the CAS Enzyme. A partial multiple-amino acid sequence alignment was generated in an effort to consider the location and nature of the observed specificity-altering mutations in the context of cyclase evolution and residues known to be important for the cyclization/rearrangement processes. The program CLUSTAL W was used to generate an alignment among the SHC from *A. acidocaldarius*, ERG7 from *S. cerevisiae*, and CAS from *Ar. thaliana* (Figure 3).

Several observations may be made from the alignment. First, one of the mutations (Ile481Thr) occurs two residues to the N-terminal side of the aspartic acid residue that is completely conserved among the triterpene cyclases and has been shown to be critical for activity (*41*, *42*). Second, two of the mutations (Ala469Val and His477Tyr) occur proximal to the active site sequence in the N-terminal direction, while two more mutations occur somewhat more distal in the N-terminal (Tyr410Cys) and C-terminal (Tyr532His) directions. Third, mutations at two of these positions have been reported previously to lead to production of lanosterol by CAS, although the mutations are not identical (Ile481Val and Tyr410Thr) (*21*, *23*). Fourth, in no case examined do the specificity-altering mutations result in the substitution of an amino acid residue found in CAS from *Ar. thaliana* with that found at the corresponding position in ERG7 from *S. cerevisiae*. Indeed, the Ala469Val and Tyr532His mutations change residues that are conserved between these two enzymes. Fifth, three of the five mutations involve changes to or from tyrosine residues.

To provide some insight into the structural basis for the altered product specificity observed with the mutants identi-

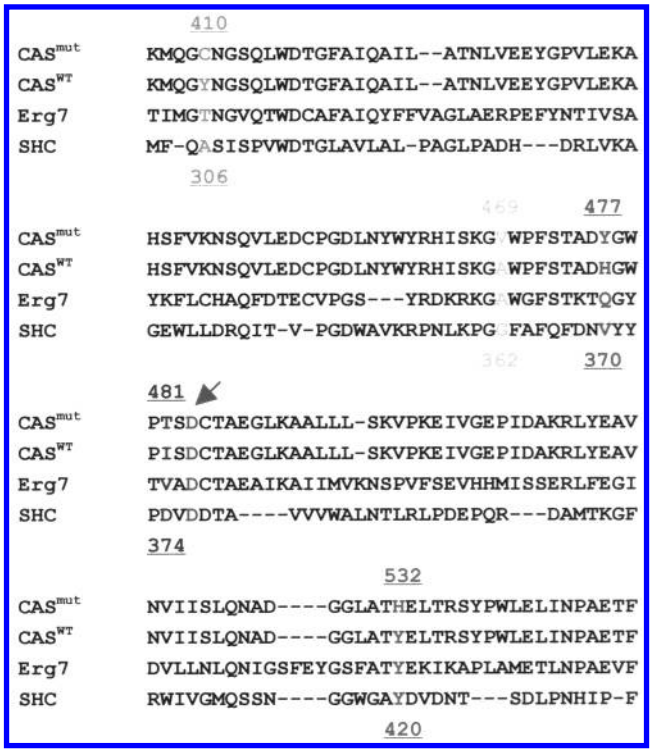

FIGURE 3: Partial multiple-sequence alignment of wild-type and mutant forms of 2,3-oxidosqualene-cycloartenol synthase from *Ar. thaliana* (CAS), wild-type 2,3-oxidosqualene-lanosterol cyclase from *S. cerevisiae* (ERG7), and squalene-hopene cyclase from *A. acidocaldarius* (SHC) generated from the program CLUSTAL W. Gaps were allowed to achieve optimum alignment. The mutants are numbered according to their position in CAS or SHC. The conserved active site aspartic acid residue (position 483 in CAS from *Ar. thaliana*) is indicated with an arrow.

fied through random mutation and selection, we generated a structural homology model of CAS. This model was constructed using the X-ray structure of SHC from *A. acidocaldarius* and the GeneMine program which assembles segments of structural and sequence homology using a fragment-matching algorithm (*12*, *13*, *37*, *38*). The GeneMine output was refined and analyzed using the ENCAD energy minimization program (*39*). We eliminated CAS amino acid residues 1−92 in generating the model because these residues are not homologous with SHC. To help relate the positions of the observed mutations to the cyclase active site, we included in the model the competitive inhibitor *N*,*N*-dimethyldodecylamine *N*-oxide (LDAO) (*43*) in a position analogous to that at which it is observed bound to SHC (*12*, *13*).

Two views of the resulting model are provided in Figure 4. The first view (A) presents the entire model structure as a ribbon diagram with the side chains of the mutated amino acids, the essential residue Asp483, and LDAO shown in space-filling form. The second view presents a closer picture of the region of the enzyme bearing the mutated residues. Here the side chains of interest and LDAO are depicted in ball-and-stick form. The model indicates that three of the residues where mutations alter product specificity (Tyr410Cys, Ile481Thr, and Tyr532His) are likely to lie within the active site as defined by the bound inhibitor LDAO. Interestingly, the model predicts that the Ala469Val and His477Tyr mutations do not change residues that are in direct contact with the bound substrate.

DISCUSSION

We have shown that a combination of random mutation coupled with in vivo selection provides an effective method of identifying single-amino acid changes that lead to alterations in the product specificity of a triterpene cyclase enzyme. Our approach and results complement and extend previous mutagenesis and directed evolution studies of triterpene cyclase enzymes and provide three important findings.

One finding of our studies is the frequency at which point mutations can alter the course of cyclization and/or rearrangement. For the particular case we have studied, oxidosqualene-cycloartenol synthase from *Ar. thaliana*, we find that point mutations inducing oxidosqualene-lanosterol cyclase activity can be generated at an impressive frequency (approximately one in 15 000). Furthermore, we believe this

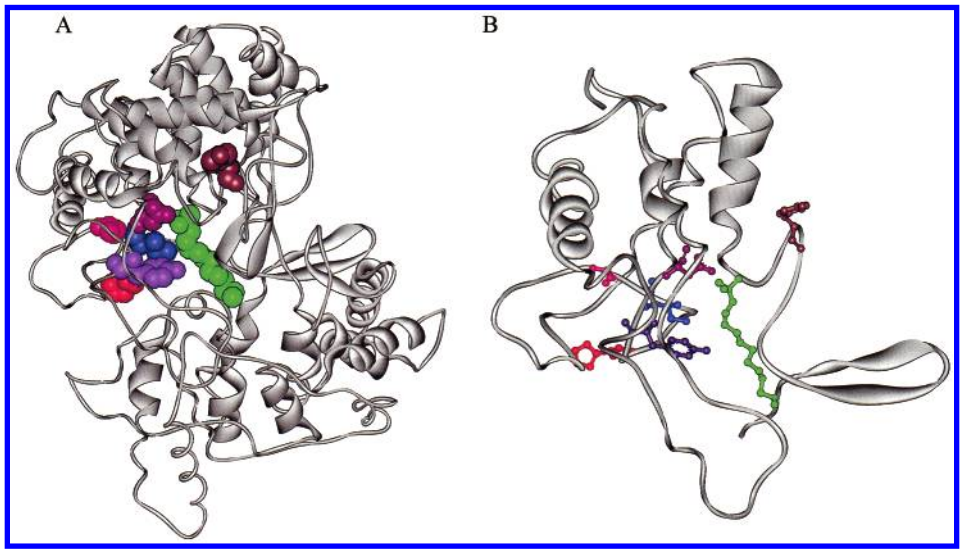

FIGURE 4: Two views of the homology model of the CAS enzyme based on the SHC X-ray structure, built using the GeneMine program. (A) Solid ribbon representation of the CAS homology model, excluding amino acid residues 1−92. (B) Local view of the mutant residues on the putative active site cavity (His477, red; Ala469, pink; Tyr532, purple; Ile481, blue; Asp483, violet; Tyr410, brown; and LDAO, green).

value represents a lower limit to the frequency at which mutations alter the product specificity of *Ar. thaliana* CAS, since our selection method did not allow us to identify mutants that catalyze the formation of products other than lanosterol.

A second finding of our studies is the identification of a new position in the *Ar. thaliana* CAS active site where mutations alter product specificity. This mutation (Tyr532His) is almost certain to occur at the active site based on the structure of *A. acidocaldarius* SHC and the CAS structural homology model we derived therefrom (*12*, *13*). Tyrosine also occurs at the homologous position in ERG7 from *S. cerevisiae* and in SHC from *A. acidocaldarius* (Figure 3). Formation of the observed monocyclic product achilleol A by the Tyr532His mutant could be rationalized on the grounds that this residue plays an important role in the procession of cyclization for triterpene cyclases generally. However, by the same token, Tyr532 mutation would not have been expected to impart oxidosqualene-lanosterol cyclase activity. The model indicates that Tyr532 lies adjacent to Ile481 in CAS, where the Ile481Thr mutation and a series of other mutations alter product specificity (*21*, *22*, *25*). In contrast, Tyr532 would appear to lie on the opposite face of the cyclizing/rearranging substrate relative to Tyr410, where the Tyr410Cys mutation as well as the previously identified Tyr410Thr mutation effects lanosterol formation (*23*). The finding that another tyrosyl residue plays an important role in oxidosqualene cyclization is consistent with the "aromatic hypothesis" promulgated on the basis of early triterpene cyclase sequence information and supported by the SHC structure, i.e., that the electron-rich aromatic side chains of Tyr, Trp, and Phe residues play important roles in the generation, shepherding, and termination of electron-poor intermediates and transition states associated with cyclization and/or rearrangement (*29*, *44*−*47*).

The third finding of our studies is the identification of oxidosqualene-lanosterol cyclase activity associated with the novel Ala469Val and His477Tyr mutants of CAS. Residues at corresponding positions in SHC do not contribute to the active site, and our CAS structural homology model indeed indicates that these residues are not in direct contact with the substrate. If the model is accurate in this regard, Ala469Val and His477Tyr would represent, to our knowledge, the first examples of nonactive site mutations that alter product specificity in a triterpene cyclase. Ala469 and His477 may be nearest neighbors to active site residues and thereby provide an indirect influence on active site structure. These effects need not be great in light of the work of Matsuda and co-workers (*21*, *22*, *25*), who showed that the product specificity of CAS could be altered by even subtle changes in the steric bulk of the active site residue Ile481.

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