Supporting information for

Enzyme Redesign: Two Mutations Co-operate to Convert Cycloartenol Synthase into an Accurate Lanosterol Synthase

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Construction of *Ath*CAS1 H477N I481V and *Ath*CAS1 H477Q I481V double mutants. The *Ath*CAS1 H477N I481V double mutant construct was created by sub-cloning a *Bgl* II fragment (~650 bp) containing the H477N/I481V mutations from the *Ath*CAS1 Y410T H477N I481V triple mutant[1] into the wild type *Ath*CAS1 *Bgl* II fragment (~8110 bp). Both original constructs were in the integrative galactose-inducible yeast expression vector pRS305GAL. DNA plasmids were excised with *Bgl* II (NEB); fragments were gel-purified with QIAquick Gel Extraction Kit (Qiagen) and ligated using Quick Ligation Kit (NEB) following the manufacturers' procedures. Ligation mixtures were transformed into DH5α competent cells, plated on LB amp plates and grown overnight at 37 °C. Transformants were then used to inoculate LB amp liquid cultures and plasmid DNA was prepared for mapping, sequencing and yeast transformation. The cloned gene was sequenced to ensure that only the desired mutations had been incorporated.

The *Ath*CAS1 H477Q I481V double mutant was generated similarly by subcloning the corresponding *Bgl* II fragment from the *Ath*CAS1 Y410T H477Q I481V triple mutant[1] into the wild-type *Ath*CAS1 *Bgl* II fragment.

Complementation assays. The *Ath*CAS1 H477N I481V and *Ath*CAS1 H477Q I481V constructs were linearized with *Bst*E II and transformed into the yeast lanosterol synthase deletion mutant SMY8[2] using the lithium acetate protocol.[3] Transformants were selected on SCD-Leu,H,E (synthetic complete medium[4] lacking leucine, 2% glucose, 13 mg/L heme, 20 mg/L ergosterol) plates. Positive transformants were streaked on YPGH (1% yeast extract, 2% peptone, 2% galactose, 13 mg/L heme, no ergosterol supplementation) plates, to induce expression and check the ability of the mutants to genetically complement the lanosterol synthase deletion. SMY8 expressing *Saccharomyces cerevisiae* lanosterol synthase[5] (*Sce*Erg7p) was used as positive control and SMY8 expressing *Arabidopsis thaliana* cycloartenol synthase[6] (*Ath*CAS1) was used as negative control. These complementation experiments revealed that both mutants supported growth without exogenous ergosterol at a rate comparable to wild type *Sce*Erg7p (colonies visible in two days) whereas *Ath*CAS1 did not.

***In vitro* assays.** The *Ath*CAS1 H477N I481V and *Ath*CAS1 H477Q I481V constructs were linearized with *Bst*E II and transformed into the yeast strain RXY6[7] (squalene epoxidase/lanosterol synthase double mutant). Transformants were selected on SCD-Leu,H,E plates. Positive transformants were used to inoculate SCD-Leu,H,E liquid cultures (10 mL). All yeast cultures were grown at 30 °C and liquid cultures were shaken at 250 rpm. Upon saturation, the 10 mL cultures were used to inoculate YPD,H,E (1% yeast extract, 2% peptone, 2% dextrose, 13 mg/L heme, 20 mg/L ergosterol) media (100 mL). When saturated, the 100 mL cultures were used to inoculate 1-L scale induction medium YPG,H,E (1% yeast extract, 2% peptone, 2% galactose, 13 mg/L heme, 20 mg/L ergosterol). Upon saturation, cells were harvested by centrifugation, resuspended in two volumes of 100 mM sodium phosphate buffer pH 6.2 and lysed using an Emulsiflex-C5 homogenizer. The cell homogenates were incubated at room temperature for 24 h with racemic oxidosqualene (1 mg/mL) solubilized in Tween 80 (0.1%). Negative controls were prepared from aliquots of the cell homogenates containing no substrate. The reactions were monitored by silica gel TLC. Aliquots of the enzymatic reaction mixtures (5 μL) were spotted on a TLC plate as thin vertical bands. A mixture of authentic lanosterol and ergosterol standards was spotted alongside. The TLC plate was air dried and then partially developed twice with ethyl ether to about one fourth the length of the plate to focus each spot to a single point. The plate was then developed with 1:1 hexane/ethyl ether. Products were visualized by staining with *p*-anisaldehyde. TLC analysis showed a triterpene alcohol spot that comigrated with the lanosterol standard (Rf 0.5 in 1:1 hexane/ethyl ether) and was well separated from the oxidosqualene substrate (Rf 0.8) and yeast sterols such as ergosterol (Rf 0.3). No oxidosqualene cyclization products were visualized in the negative control assays.

Product isolation and characterization. The *in vitro* assays were quenched with two volumes of ethanol and the cellular debris was removed by centrifugation. The ethanol was eliminated by rotary evaporation and the remaining aqueous suspension was extracted with methyl *tert*-butyl ether (MTBE). The combined MTBE extracts were washed with brine and then concentrated to dryness by rotary evaporation. Aliquots of these crude extracts were analyzed by GC, GC-MS, and 500 MHz ^{1}H NMR.

Triterpene alcohol fractions were purified from crude extracts by silica gel column chromatography using gradients of ethyl ether/hexane. Column separation was monitored by TLC. Products were identified by 500 MHz ^{1}H NMR analysis and by GC and GC-MS analysis of the trimethylsilyl (TMS) ether derivatives. Fraction A (elution with 2% ether in hexanes) gave squalene (*m/z* 410). Fraction B (elution with 5% ether in hexanes) gave 2,3-oxidosqualene (*m/z* 426). Fraction C (elution with 5-10% ether in hexanes) gave triterpene alcohols (lanosterol, cycloartenol, and/or parkeol as TMS ethers, *m/z* 498). Fraction D (elution with 20% ether in hexanes) gave ergosterol-TMS (*m/z* 468) and minor amounts of ergosterol-TMS derivatives (*m/z* 466, 468, and 470). A polar fraction was eluted with 50-100% ether in hexanes. Apart from fraction C, no fraction contained peaks corresponding to *m/z* 498.

Product ratios were determined by GC-FID quantitation of the partially purified fractions and confirmed by GC-FID quantitation of the MTBE crude extracts. Integration of well-resolved ^{1}H NMR peaks in the column purified material provided similar product ratios.

Activity Comparison. It is notoriously difficult to measure K_m values in the presence of detergents because the concentrations of substrate and enzyme are distorted by the biphasic aqueous and micellar system. Although most of the enzyme and substrate are probably constrained to the restricted volume of the micelles, the soluble proportion is not readily determined. We consequently compared the catalytic competence of the native and mutant enzymes using homogenate assays with substrate at a

concentration (1.2 mM) well above the literature K_m of 25-125 μM described for several plant cyclases.[8-10]

Duplicate samples of *Ath*CAS1 H477N I481V and wild type *Ath*CAS1 expressed in RXY6 were grown under identical conditions as described above and collected by centrifugation. Each yeast strain was suspended in 2 volumes of 100 mM sodium phosphate buffer pH 7 and lysed using an Emulsiflex-C5 homogenizer. After lysis, 100 mM sodium phosphate buffer pH 7 was added to make a 20% slurry. A solution of racemic 2,3-oxidosqualene and Triton X-100 was added to the homogenate aliquots (350 μL) to give a final concentration of 1 mg/mL substrate and 0.1% Triton X-100. After 0.5, 1, 3, 5, and 10 hours, the reactions were terminated by adding two volumes of ethanol. Cholesterol (8 μg) was added to each reaction as an internal standard. The denatured protein was removed by centrifugation and the supernatant was concentrated to dryness under a nitrogen stream. The residue was resuspended in ethyl ether, loaded onto a short silica gel plug and eluted with ethyl ether. Extracts were evaporated to dryness and residues were derivatized with BSTFA-pyridine (1:1) and analyzed by GC-FID. The native enzyme generated 20 μg cycloartenol/(h × mg yeast) (n=2, mean error 2 μg /(h × mg yeast), and the H477N I481V mutant produced 12 μg lanosterol/(h × mg yeast) (n=2, mean error 2 μg /(h × mg yeast).

These experiments indicate that the H477N I481V mutant has about half the activity of the wild-type *Ath*CAS1 enzyme. This mutant has quite good efficiency considering that many triterpene cyclase mutants have very low efficiency compared to wild-type enzyme.[11]

Since the substrate concentration should be well above the K_m and since the micellar distortions of the functional volume could provide even higher local substrate concentrations, the mutant's modest decrease in catalytic efficiency is probably primarily a k_{cat} effect.

GC and GC-MS analysis. Gas chromatography (GC) analysis was performed on an Agilent 6890 instrument equipped with an Rtx-35MS column (30 m, 0.25 mm i.d., 0.10 μm df; Restek, Bellefonte, PA). GC conditions: inlet and FID-detector temperatures were 280 °C; the oven was kept isothermal at 260 °C; split injection was used with a ratio of 40:1; helium was the carrier gas at a constant flow of 1 mL/min.

GC-MS analysis was performed on an Agilent 6890 gas chromatograph interfaced to a 5973 MSD using electron impact at 70 eV. The GC column and conditions were identical to those for GC analysis.

Triterpene alcohol products were converted to their corresponding trimethylsilyl (TMS) ethers as follows. After evaporation of solvent the sample residue was derivatized by treatment with 100 μL of dry pyridine and 100 μL of bis(trimethylsilyl)trifluoroacetamide (BSTFA). The samples were then sealed and left at 37 ºC for 2 h. The resulting solution was used directly for GC or GC-MS.

Authentic standards of lanosterol and parkeol were derivatized and analyzed under the same conditions for comparison. Retention times: TMS-lanosterol, 15.9 min; TMS-parkeol, 17.8 min.

NMR analysis. Proton (^{1}H) NMR spectra were collected at 500 MHz on a Bruker Avance spectrometer equipped with a 5 mm inverse geometry probe. Tetramethylsilane (TMS) was used as internal standard. All spectra were collected at 25 ºC. Deuterated chloroform ($CDCl_3$) was filtered through basic alumina prior to use and all samples were prepared in 5 mm glass tubes (Wilmad Glass Co., Inc.).

The triterpene alcohol fraction of *Ath*CAS1 H477N I481V had the following resolved ^{1}H NMR signals characteristic of lanosterol:[12] δ 0.690 (3H, s, H-18), 0.811 (3H, s, H-29), 0.982 (3H, s, H-19), 1.001 (3H, s, H-28), and 3.236 (1H, dd, H-3α); and the following resolved signals for parkeol:[13] δ 0.647 (3H, s, H-18) and 0.737 (3H, s, H-30).

The triterpene alcohol fraction of *Ath*CAS1 H477Q I481V had the following ^{1}H NMR resolved signals characteristic of lanosterol:[12] δ 0.690 (3H, s, H-18), 0.811 (3H, s, H-29), 0.982 (3H, s, H-19), 1.001 (3H, s, H-28), and 3.235 (1H, dd, H-3α); and the following resolved signals for parkeol:[13] δ 0.647 (3H, s, H-18) and 0.737 (3H, s, H-30).

Computational Analysis. The homology model of *Ath*CAS1 was built as described previously[1] using the latest software release of MOE (Chemical Computing Group Inc, Montréal, Québec, Canada), version 2004.03. The *Aac*SHC crystal structure (PDB entry 2SQC, resolution 2.00 Å) obtained from the Protein Data Bank (PDB) was used as template. Late in the course of our work the crystal structure of human lanosterol synthase (PDB entry 1W6K, resolution 2.1Å) was reported.[14] *Ath*CAS1 and human lanosterol synthase are 44% identical while *Ath*CAS1 is only 22% identical to *Aac*SHC, the original template for the homology model. Therefore, the model was further refined by using the new structural information. During optimization of the model no major structural changes were observed for active site residues, and refinement consisted only on fine-tuning the coordinates of active-site side chain atoms. Modeling of mutants and manual substrate docking was carried out with the modeling package Moloc (Gerber Molecular Design, Basel, Switzerland).

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