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Structural and Biochemical Studies of a Biocatalyst for the Enzymatic Production of Wax Esters

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Abstract

Wax esters are high-value products whose enzymatic synthesis is of increasing biotechnological interest. The fabrication of cell factories that mass-produce wax esters may provide a facile route towards a sustainable, and environment-friendly approach to a large-scale process for this commodity chemical. An expedient route for wax-ester biocatalysis may be facilitated by the action of enzymes termed wax ester synthases/diacylglycerol acyltransferases (WS/DGAT), which produce wax esters using fatty acids and alcohols as a precursor. In this work, we report the structure for a member of the WS/DGAT superfamily. The structural data in conjunction with bioinformatics and mutational analyses allowed us to identify the substrate binding pockets, and residues that may be important for catalysis. Using this information as a guide, we generated a mutant with preference towards shorter acyl-substrates. This study demonstrates the efficacy of a structure-guided engineering effort towards a WS/DGAT variant with preference towards wax esters of desired lengths.

Graphical Abstract

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Supporting Information

The Supporting Information includes the following: (1) table with primers used for amplification of wild type Ma-WS/DGAT and generation of mutants, (2) table with data collection, phasing and refinement statistics for Ma-WS/DGAT crystal structure, (3) SDS-PAGE image of wild type Ma-WS/DGAT and mutants, (4) Coenzyme A standard curve, (5) plots with observed rates for wild type Ma-WS/DGAT and mutants, (6) GC/MS and LC/MS data for the detection of hexyl-palmitate and tripalmitate as confirmation of Ma-WS/DGAT activity, (7) sequence alignment of Ma-WS/DGAT, Ab-WS/DGAT, FsTRI3 and AtHCT, (8) superposition of Ma-WS/DGAT with fsTRI3 and AtHCT, and (9) sequence logo generated by WebLogo (<http://weblogo.berkeley.edu/>) using as input the curated seed alignment of PFAM for wax synthases (PF03007).

Notes

The authors declare no competing financial interest.

Keywords

Wax ester synthase; Diacylglycerol acyltransferase; WS/DGAT; Wax esters; Biodiesel; Crystal structure; *Marinobacter aquaeolei* VT8

INTRODUCTION

Wax esters have widely been used in the cosmetics, lubricant, candle, and food industries since the 19th century.¹ Up until the 1960s, the main source of wax esters was oil from sperm whales, until their endangerment resulted in a global ban on whaling.² Since the Endangered Species Act in 1973 and the ban on sperm oil use, alternative sources of wax esters have been sought. Currently, wax esters are extracted from plants (e.g. jojoba oil) or are chemically synthesized from expensive precursors.^{3–4} Hence, there is a strong demand for the development of large-scale processes for the production of low cost wax esters. Towards that end, there has been increasing focus in the development of methods for the enzymatic biosynthesis of wax esters. Current methods center on the use of enzymes immobilized on solid supports, as well as the use of cellular ‘factories’ engineered to produce the desired wax esters.^{4–8}

Wax esters are esters of long chain fatty acids and long chain alcohols that are formed *in vivo* by the condensation of a fatty alcohol and a fatty acyl-Coenzyme A (acyl-CoA), a reaction catalyzed by acyltransferases termed wax ester synthases (WS, EC 2.3.1.75). These acyltransferases are largely found in bacteria, plants and animals, and can also catalyze the transesterification of acyl-CoAs with diacylglycerols (DAGs), an activity known as diacylglycerol *O*-acyltransferase (DGAT, EC 2.3.1.20).^{9–13} In bacteria, many of the WSs that have been characterized thus far are bifunctional, and encode both WS and DGAT activities.^{13–16} Although a few bifunctional WS/DGATs from plants and marine protists have been characterized, there is a dearth of information about the large number of predicted WS/DGAT sequences in eukaryotic genomes that have yet to be experimentally validated.^{14, 17–20}

Bacterial WS/DGATs are presumptively promiscuous enzymes as homologs from different species show preference for different acyl chain length and/or degree of saturation.^{21–23} This promiscuity has been exploited in the biotechnological utilization of different WS/DGAT homologs for the *in vivo* production of wax esters and biodiesel (esters consisting of medium chain fatty acids and ethanol).^{5, 8, 24–25} Currently, the most well studied WS/

DGATs are those from *Acinetobacter baylyi* ADP1 (Ab-WS/DGAT) and *Marinobacter aquaeolei* VT8 (Ma-WS/DGAT).^{12, 14, 21–22, 26–27} These enzymes have been utilized for the *in vivo* production of biodiesel and other wax esters by several laboratories, and engineering efforts to alter their substrate specificity have been an area of significant focus.^{5, 23, 26, 28–33} There is currently no structure available for any member of this enzyme family in the Protein Data Bank (PDB), which limits engineering efforts aimed at altering substrate scope for a given WS/DGAT.

Bacterial WS/DGATs share low overall sequence similarity not only with eukaryotic WS/DGATs, but also with other bacterial homologs, both across and within species.^{13–14} Despite the overall low sequence similarity, all WS/DGATs share a conserved HHXXXDG motif, which is also found in other acyltransferases, such as the condensation domains of non-ribosomal peptide synthases, chloramphenicol acetyltransferase, and polyketide-associated proteins (Pap) from *Mycobacteria*.^{34–35} In each of these acyltransferases, the conserved motif contains a catalytic His that has been proposed to act as the general base that abstracts a proton from the alcoholic substrate hydroxyl to generate a nucleophilic alkoxide (Scheme 1).^{34–37} Attack of the alkoxide onto the carbonyl carbon of the acyl-CoA thioester produces a tetrahedral intermediate, which subsequently collapses to yield the acylated alcohol and free CoA. In the case of chloramphenicol acetyltransferase and PapA5 from *Mycobacterium tuberculosis*, the second His in the HHXXXDG motif is proposed to be the general base.^{35–37} However, in bacterial WS/DGATs, the identity of the catalytic His has yet to be established. *In vitro* studies and mutational analyses performed with WS/DGATs from *Acinetobacter baylyi* ADP1 and *Marinobacter aquaeolei* VT8 showed that mutation of either of the two His severely affected the activity of the enzyme, suggesting that both are important for catalysis.^{13–14} Mutation of the conserved Asp in this motif, decreased, but did not abolish the activity of the enzyme suggesting a structural, rather than catalytic role.^{14, 38}

Here, we report the first crystal structure of any WS/DGAT, that from *Marinobacter aquaeolei* VT8 (Ma-WS/DGAT, homolog WS1, UniProt accession number A1TX06). The structural data in conjunction with structure-guided mutational analysis allowed assignment of the roles for residues in the conserved HHXXXDG motif, and provide a molecular framework for identifying the binding sites for each of the two substrates. We also utilized the structure to guide engineering experiments aimed at altering the substrate scope of the enzyme. Our studies demonstrate that structure-guided engineering may be an expedient route for producing WS/DGAT variants with different product profiles, which is an area of immense interest in biotechnology.

EXPERIMENTAL SECTION

Chemical and Reagents

All chemical reagents were purchased from Sigma-Aldrich unless otherwise noted. All materials used for protein production and purification were purchased from GE Healthcare.

Cloning and Site-Specific Mutagenesis

Ma-WS/DGAT (GenBank accession number WP_011783747 – locus NC_008740.1) was amplified using the PCR with template genomic DNA of *Marinobacter aquaeolei* VT8 (DSM 11845) and primers designed based on the published sequence. The gene was inserted into the pET His6-Sumo-TEV LIC cloning vector (2S-T) (Addgene plasmid # 29711), and this plasmid was used as template for the generation of the site-specific mutants by PCR (primers shown in Table S1). Sanger sequencing was used to confirm the integrity of all recombinant plasmids (ACGT, Inc.).

Protein Expression and Purification

Expression vectors bearing wild type or mutant Ma-WS/DGAT were transformed into *E. coli* Rosetta 2(DE3) cells for heterologous protein production. A 4 mL fresh starter culture was inoculated in 1 L Luria-Bertani growth medium supplemented with 100 µg/mL ampicillin and 25 µg/mL chloramphenicol. The culture was grown at 37 °C until the absorbance at 600 nm reached 0.6 – 0.8, at which point protein production was induced by addition of 0.3 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). The culture was then cooled to 18 °C and grown with shaking for an additional 18 h. Cells were collected by centrifugation, resuspended in Buffer A (20 mM Tris-HCl, pH 8.0, 500 mM NaCl, 10% glycerol), and lysed by multiple passages through a C5 Emulsiflex (Avestin) cell homogenizer. Following centrifugation of the lysate, the supernatant was applied to a 5 mL His-Trap (GE Biosciences) column that was previously equilibrated with Buffer B (20 mM Tris-HCl, pH 8.0, 1 M NaCl, 30 mM imidazole, 10% glycerol). The column was washed with 20 mL of buffer B, 20 mL of buffer B supplemented with 5 mM MgCl₂ and 5 mM ATP, and 15 mL of buffer C (20 mM Tris-HCl, pH 8.0, 1 M NaCl, 50 mM imidazole, 10% glycerol). Elution of the bound, His6-SUMO-tagged protein was carried out using a gradient of increasing imidazole concentration (to a final concentration of 200 mM). Fractions containing protein of the highest purity (as determined by sodium dodecyl sulphate - polyacrylamide electrophoresis, SDS-PAGE) were pooled, and the His₆-SUMO-tag was removed by overnight incubation with tobacco etch viral protease, followed by dialysis against Buffer D (20 mM Tris-HCl, pH 8.0, 1 M NaCl, 1 mM DTT, 10% glycerol) at 4 °C. The dialyzed samples were re-applied to a 5 mL His-Trap (GE Biosciences) column to remove the His6-SUMO-tag, and pure fractions of the tag-excised protein were further purified using size exclusion chromatography (SEC) (Superdex Hiload 200 16/60) in a buffer of 20 mM Tris-HCl, pH 8.0, 1 M NaCl. Samples were concentrated using a 10,000 Da molecular weight cutoff Amicon centrifugal filters. Selenomethionine-labeled (SeMet) Ma-WS/DGAT was produced by repression of methionine biosynthesis in defined media prior to protein induction, and was purified as described above. All proteins were flash frozen in liquid nitrogen and stored at –80 °C.

For activity studies, Ma-WS/DGAT wild type and mutants were purified as described above with the following modification: After cleavage of the His₆-SUMO-tag and the second His-affinity column, all samples were passed through a PD-10 desalting column (GE Healthcare), instead of SEC, for buffer exchange (20 mM Tris-HCl, pH 8.0, 1 M NaCl). The purity of all samples was assessed by SDS - PAGE analysis (Figure S1), and protein concentrations were determined by NanoDrop (Thermo Fisher Scientific) analyses using the

molecular weights and extinction coefficients calculated by the ProtParam tool (ExPASy server) (Table S2).³⁹

Crystallization

Initial crystallization conditions were determined by the sparse matrix sampling method using commercial screens. Crystals of Ma-WS/DGAT were grown using the hanging vapor drop diffusion method. Briefly, 1 μ L of protein at 4 mg/mL concentration was incubated with 1.2 molar equivalents of palmitoyl-CoA (C16-CoA), mixed with 2 μ L of precipitant solution (0.2 M L-Proline, 0.1 M MES, pH 7.0, 8% PEG 3350), and equilibrated over a well containing the same precipitant solution at 9 °C. Crystals took 1 month to grow, and were used to seed subsequent optimization trays. After several rounds of optimization and seeding, diffraction-quality crystals were obtained at 6 mg/mL protein concentration in 0.2 M L-Proline, 0.1 M MES, pH 7.0, 12% PEG 3350 at 16 °C. The crystals were sequentially soaked in precipitant solution supplemented with increasing concentrations of 10, 20 and 30% ethylene glycol prior to vitrification by direct immersion in liquid nitrogen. SeMet crystals were grown by seeding with seeds from native crystals at 2 mg/mL protein concentration in 0.2 M L-Proline, 0.1 M MES, pH 7.0, 12% PEG 3350 at 16 °C. Attempts to soak the crystals with palmitoyl-CoA (C16-CoA), hexanoyl-CoA (C6-CoA), CoA and hexanol (C6-OH) were not successful.

Data Collection, Phasing and Structure Determination

X-ray diffraction data were collected at Life Sciences Collaborative Access Team (LS-CAT), Sector 21, Argonne National Laboratory. All data were indexed, integrated and scaled using either HKL2000 or AutoProc.^{40–43} Initial crystallographic phases were determined using single wavelength anomalous diffraction data collected from crystals of SeMet-labeled Ma-WS/DGAT at the Se absorption edge ($\lambda=0.97872$ Å) to a diffraction limit of 2.9 Å. Heavy atom sites were located using the Hybrid Substructure Search submodule of the Phenix program suite,^{44–46} and were subsequently imported into the AutoSol wizard of Phenix for maximum likelihood phasing,⁴⁷ yielding an initial figure of merit of 0.369. A partial model of the structure was generated using automated methods as implemented in RESOLVE, and this initial model was further extended using the Phenix Autobuild wizard.⁴⁸ The resultant model was used as search probe for the determination of the native crystal structure by molecular replacement as implemented in the Phenix program suite.⁴⁶ The resultant solutions were subsequently used as starting models for several rounds of automated model building using Phenix Autobuild and Buccaneer,^{48–51} followed by rounds of manual rebuilding using Coot,⁵² and refinement using either Phenix Refine or REFMAC5.^{53–54} Water molecules were added during refinement with Phenix Refine, and confirmed by manual inspection. In all cases, the quality of the in-progress model was routinely monitored using both the free R factor and MolProbity for quality assurance.^{46, 55} All crystallographic data collection and refinement statistics are provided in Table S3.

Wax Ester Synthase Activity Assay

The wax ester synthase (WS) activity of the wild type enzyme and mutants was investigated by gas chromatography-mass spectroscopy (GC/MS). 200 μ L reactions containing 5.0 μ M Ma-WS/DGAT, 50 μ M palmitoyl-CoA (C16-CoA) or 100 μ M hexanoyl-CoA (C6-CoA) and

1 mM 1-hexanol (C6-OH) were incubated at 30 °C for 0.5 h and stopped by addition of 200 μ L chloroform. They were subsequently vortexed and spun down, and the chloroform phase was extracted and analyzed by GC/MS. All reactions were performed in 50 mM NaH₂PO₄-NaOH, pH 7.5, 300 mM NaCl and 1.45% (final concentration) dimethyl sulfoxide (DMSO). The activity of the mutants was expressed as “percentage of wax esters produced” in respect to the wild type reaction (considered as 100%). For this purpose, the area under the wax ester peak of the wild type reaction was considered as 100%, and the areas under the wax ester peaks of the mutants were converted to “% of activity” with the following equation: % of activity = (peak area of mutant) x 100 / (peak area of wild type). Control reactions without addition of the enzyme were also analyzed. Results are means of duplicate experiments.

For the competition assays, reactions were prepared as described above with the following modifications: 1) 50 μ M C16-CoA and 50 μ M C6-CoA were both added in the reactions and the reactions were initiated by addition of 1 mM C6-OH, and 2) 100 μ M dodecanol (C12-OH) and 100 μ M C6-OH were both added in the reactions and the reactions were initiated by addition of 100 μ M C6-CoA. The sum of the areas under the peaks for 1) hexyl-palmitate and hexyl-caproate and 2) dodecyl-caproate and hexyl-caproate respectively, was considered as the total amount of wax esters produced (100%) and the relative abundance of each peak is reported as % of hexyl-palmitate, % of hexyl-caproate and % of dodecyl-caproate produced by each enzyme. Results are means $\pm$ SEM of duplicate experiments.

Diacylglycerol Acyltransferase Activity Assay

The diacylglycerol acyltransferase (DGAT) activity of the wild type enzyme was investigated by liquid chromatography-mass spectroscopy (LC/MS). 200 μ L reactions containing 5.0 μ M Ma-WS/DGAT, 50 μ M palmitoyl-CoA (C16-CoA) and 200 μ M 1,2-dipalmitoyl-sn-glycerol were incubated at 30 °C for 1 h and stopped by addition of 200 μ L chloroform. They were subsequently vortexed and span down, and the chloroform phase was extracted and analyzed by LC/MS. All reactions were performed in 50 mM NaH₂PO₄-NaOH, pH 7.5, 300 mM NaCl and 1.45% (final concentration) DMSO.

Gas Chromatography-Mass Spectrometry (GC/MS)

The samples were analyzed using a GC/MS system (Agilent Inc, CA, USA) consisting of an Agilent 7890 gas chromatograph, an Agilent 5975 MSD and an Agilent 7683B autosampler. Gas chromatography was performed on a ZB-1MS (30 mm $\times$ 0.32 mm I.D. and 0.25 μ m film thickness) capillary column (Phenomenex, CA, USA). The inlet and MS interface temperatures were set to 280 °C, and the ion source temperature was adjusted to 230 °C. An aliquot of 1 μ L was injected in a splitless mode (purge time 0.5 min). The helium carrier gas was kept at a constant flow rate of 2.3 mL/min. The temperature program was: 2 min isothermal heating at 180 °C, followed by an oven temperature increase of 15 °C/min to 330 °C and a final 10 min at 330 °C. The mass spectrometer was operated in positive electron impact mode (EI) at 69.9 eV ionization energy at m/z 33–500 range. Mass spectra were recorded in the combined scan/SIM mode. For SIM mode m/z 340, 257 (hexyl-palmitate), 117 (hexyl-caproate) and 537 (dodecyl-caproate) were tracked. Hexyl- and dodecyl-caproate were confirmed with NIST08 commercial database (NIST, MD, USA)

while hexyl-palmitate was confirmed by high resolution GC/MS. The mass spectrum of target compound was evaluated using the MSD Chemstation E.02.01.1177 software (Agilent, Palo Alto, CA, USA).

Liquid Chromatography-Mass Spectrometry (LC/MS)

The sample was analyzed by Q-Exactive MS system (Thermo. Bremen, Germany), and software Xcalibur 4.1.31.9 was used for data acquisition and analysis. Mass spectrum was acquired under positive ESI with the following settings: sheath gas flow rate, 49; aux gas flow rate: 12; sweep gas flow rate, 2; spray voltage, 3.5 kV; capillary temp, 259 °C; Aux gas heater temp, 419 °C. The resolution was set to 70,000. The AGC target was 1E6 with a maximum injection time of 50 ms.

Kinetic Analysis of Wax Ester Synthase activity for Wild Type and Mutants

For kinetic analysis of wild type Ma-WS/DGAT and mutants (Gly25→Val and Ala144→Phe), the progress of the reaction was monitored continuously by measuring the release of coenzyme A (CoA), using a method previously described.^{21–22, 56–57} Briefly, the free thiol of CoA was quantified with 5, 5-dithio-bis-(2-nitrobenzoic acid) (DTNB), a reagent that reacts with thiol groups to produce 2-nitro-5-thiobenzoate anion (TNB²⁻), which absorbs at 412 nm.^{56–57} The reactions were run in real-time at 25 °C in 200 µL microplates. The reaction mixture (200 µL) consisted of 50 mM NaH₂PO₄ – NaOH (pH 7.5), 300 mM NaCl, 0.2 mg/mL DTNB, 4 mM C6-OH, 4% DMSO (final concentration in all reactions), appropriate amount of purified recombinant enzymes and different concentrations of either C6-CoA or C16-CoA. Assay reactions were pre-incubated at 25 °C for 5 min before they were initiated by the addition of the enzyme pre-mixed with C6-OH. The release of free CoA was monitored for additional 15 min at 412 nm on a microplate reader Epoch 2 (BioTek). Control reactions without addition of the corresponding acyl-CoA were also included and subtracted from the enzyme reactions.

Initial reaction velocities were calculated in Excel (Microsoft, Redmond, WA) by obtaining the slope from the best-fit line for a time course of the reaction during which substrate depletion never exceeded 10%. The amount of CoA formed per s was subsequently determined by using a CoA standard curve (Figure S2). This standard curve was generated by measuring the absorbance at 412 nm of known concentrations of CoA in 50 mM NaH₂PO₄ – NaOH (pH 7.5), 300 mM NaCl, 0.2 mg/mL DTNB and 4% DMSO (200 µL final volume) after a 10 min incubation. Based on the initial reaction rates, the apparent K_M and V_{max} values were determined using the Michaelis - Menten function of Origin (OriginLab, Northampton, MA). In cases with high K_M values where saturation of the enzyme could not be achieved due to limited solubility of the substrates in the reaction buffer, the apparent K_M and V_{max} values could not be determined, but the k_{cat}/K_M values were obtained by plotting the observed rates ($k_{obs} = V_0 / [E]$) at four different substrate concentrations below the experimentally suggested K_M (Figure S3). In the case of C16-CoA, the detection limit of the assay (~3 µM of CoA) (Figure S2) did not permit the measurement of the initial reaction rates (less than 10% of substrate consumed) at concentrations below 30 µM, and the maximum observed rate was considered as the apparent k_{cat} ($k_{cat,app}$). Results are means ± SEM of duplicate experiments.

Amino Acid Conservation Analysis

The conservation of amino acids in Ma-WS/DGAT was analyzed by utilizing the ConSurf server,^{58–59} a bioinformatics tool that analyzes the conservation of amino acids in a protein taking into account the evolutionary relationships between homologs of the same family. The homologs were collected from UNIREF90 using PSI-BLAST as the search algorithm. The settings were as follows: 90% maximum identity between sequences, 35% minimum identity between sequences, total of 500 closest sequences. The retrieved sequences were aligned using ClustalW, and a Bayesian method was used for the calculation of the conservation scores. There were 419 PSI-BLAST hits, but only 396 of which were unique, and were used for the calculation.

RESULTS AND DISCUSSION

Ma-WS/DGAT Activity

The WS/DGAT homolog from *Marinobacter aquaeolei* VT8 (Ma-WS/DGAT) used in this study has previously been characterized for alcohol substrate promiscuity.^{21–22} Nonetheless, we confirmed both the WS and DGAT activities of the enzyme using recombinant purified Ma-WS/DGAT. The enzyme was able to produce hexyl-palmitate and tripalmitate when palmitoyl-CoA and hexanol or 1,2-dipalmitoyl-sn-glycerol, respectively, were provided as substrates (Figure S4).

Crystal Structure of Ma-WS/DGAT

Ma-WS/DGAT (PDB ID: 6CHJ) was crystallized as a dimer at 2.4 Å in space group P2₁ showing a structure consisting of N-terminal and C-terminal domains, connected via a helical linker (Figure 1a). The N-terminal domain is composed of a mixed β -sheet (strands β 1, β 4, β 5, β 6, β 11) flanked by 4 α -helices (helices α 1– α 4), and a small antiparallel β -sheet (strands β 2 and β 3). The C-terminal domain consists of a mixed β -sheet (strands β 7– β 10 and β 12– β 13) that on one side is covered by 4 α -helices (α 6, α 7, α 8, α 10), and on the other side it faces helix α 9 and the N-terminal domain. The two domains are connected by helix α 5 (residues 200–214). Disordered regions with poor electron density were built with caution, however, there are still small regions of unassigned density. The final model is missing residues 169–198, 219–225, 296–308 and 341–348 from chain A, and residues 169–190, 217–225 and 296–308 from chain B. This high degree of disorder and flexibility may provide one reason why the crystals took long to grow (about one month).

The crystal structure reveals the presence of a long cavity that is formed at the core of the N-terminal domain (pocket 1) and extends to the interface of the two domains (pocket 2) (Figure 1b). The conserved HHXXXDG motif is located on a loop that links strand β 6 with helix α 4 with His136 positioned to face towards pocket 2, while His135 is directed away from this cavity (Figure 1a–c). Based on the orientation of the two conserved His, it is likely that His136 is catalytic while His135 plays a structural role. This notion is further supported by the fact that His135 is within hydrogen bonding distance from the highly conserved Glu15, the side chain of Ser137 and the amide nitrogens of His136 and Ser137 (Figure 1c). Disruption of these interactions would explain why, in prior studies, enzyme activity was severely affected when the first His was substituted by Leu in the WS/DGAT from

Acinetobacter baylyi ADP1 or by Ala in a different WS/DGAT homolog from *Marinobacter aquaeolei* VT8 (WS2, UniProt accession number A1U572).^{13–14} In addition to His135, the crystal structure suggests that Asp140 also plays a structural role. The side chain of Asp140 is involved in hydrogen bonding interactions with the first three residues of helix $\alpha 4$ (Figure 1d). Presumably, disruption of these interactions would affect the structural integrity of the loop where the catalytic His135 is located and would account for the significant loss of activity when by an Ala substitution at this conserved Asp.^{14, 38}

In one of the two chains in the non-crystallographic dimer, there is unassigned electron density adjacent to His136. Attempts to fit glycerol, MES and PEG were not successful as these ligands were too small to justify the observed density. It is possible that some other ligand co-purified with the enzyme, but the quality of the density is not sufficient for a conclusive assignment.

Amino Acid Residues Conservation

The evolutionary conservation of amino acids across WS/DGATs was calculated and mapped onto the crystal structure of Ma-WS/DGAT. This amino acid conservation can provide useful information about functionally important residues, especially in protein families like the WS/DGAT that include homologs which share a low overall sequence similarity. In total, 396 sequences with sequence identity of 35–90% to Ma-WS/DGAT were aligned, and the conservation score as calculated by ConSurf server^{58–59} was mapped on the crystal structure of Ma-WS/DGAT (Figure 2). Most of the highly conserved residues are located on regions that are important for the structural integrity of the enzyme. In addition to the conserved motif, only a few highly conserved residues line the enzyme cavity. These residues are located in a “ring” (Figure 2) around where the catalysis is expected to take place and may be involved in orienting the substrates and stabilizing the tetrahedral intermediate. This low conservation of the cavity lining amino acid residues could justify the differences in substrate specificities between different homologs and could be exploited for structure-based engineering of Ma-WS/DGAT towards the design of variants with different substrate specificities.

Substrate Binding Pockets

The orientation of the conserved motif HHXXXDG suggests that the fatty alcohol or DAG substrates bind either in pocket 1 or pocket 2. Structural comparison of Ma-WS/DGAT with other acyltransferases, such as the TRI3 trichothecene 15-*O*-acetyltransferase from *Fusarium sporotrichioides* (FsTRI3) (PDB ID 3FP0, Z-score 18.9 and RMSD 4.6 Å as calculated by the Dali server) (Figure 3a),^{60–61} suggests that the acyl acceptor substrates bind in pocket 2. This is in agreement with mutational studies where mutations at residues that reside in pocket 2 altered the alcohol substrate specificity of Ma-WS/DGAT.^{26, 62} In these studies, substitutions at Leu356, Ala360 and Met405 with either larger or smaller hydrophobic residues resulted in Ma-WS/DGAT variants with different selectivity towards small, branched and aromatic alcohols. It is notable that all three residues are not highly conserved (Figure 4); in nature, there are WS/DGAT homologs that have several different amino acids in these positions (4–5 conservation score in the scale of ConSurf).⁵⁸ A closer examination of pocket 2 (Figure 4) reveals the overall low conservation of the pocket lining amino acid

residues, and provides the framework for future structure-based engineering of Ma-WS/DGAT towards the design of variants with different alcohol specificities.

Further structural comparison of Ma-WS/DGAT with other acyltransferases suggests that the phosphoadenosine moiety of acyl-CoA (Scheme 2) is exposed to the solvent and the pantetheine portion is extended into the active site with the thioester positioned close to His136 and the acyl chain buried in pocket 1 (Figure 3c). In this binding mode, the acyl chain is shielded from the solvent and the thioester is positioned next to the catalytic His136 and the acyl acceptor. Since pocket 1 is about 24 Å long, Ma-WS/DGAT could accommodate acyl chains of 19–20 carbons long if bound in a fully extended conformation. In comparison with other CoA dependent transferases that share the same fold, the pocket formed at the core of the N-terminal domain reflects the size and shape of the CoA activated substrate. In the active site of hydroxycinnamoyl-CoA:shikimate hydroxycinnamoyltransferase from *Arabidopsis thaliana* (AtHCT) (PDB ID 5KJT, Z-score 16.0 and RMSD 4.9 Å as calculated by the Dali server),^{60, 63} *p*-coumaroyl-CoA is bound in a similar mode, and the size of the pocket is sufficient to accommodate the cognate ligand (Figure 3b). Likewise, FsTRI3 acetyltransferase⁶¹ lacks a pocket at the core of the N-terminal domain as the substrate acetyl group is small and fits in the pocket formed at the interface of the two domains (Figure 3a).

A superposition of the structures of FsTRI3 and AtHCT with Ma-WS/DGAT reveals the molecular features that dictate the formation, size and shape of pocket 1 in the latter enzyme. These features include shortening of strand β 11 by introduction of a Pro residue at the C-terminal end of the strand, introduction of bulky hydrophobic residues such as Phe467 in FsTRI3 and Tyr40 in AtHCT to define the length of the binding pocket, and use of smaller hydrophobic residues such as Gly, Ala and Val to line the pocket of Ma-WS/DGAT (Figure 3d–f). In addition to these amino acid substitutions, the relative disposition of helix α 4 and the β -sheet of the N-terminal domain is different among these acyltransferases and also results in the formation of differently sized binding pocket (Figure S6).

To further investigate whether pocket 1 encompasses the acyl chain of palmitoyl-CoA, we generated site-specific variants and compared their activity with that of the wild type Ma-WS/DGAT (Table 1). We selected Gly25 and Ala144 to be mutated because of their position in pocket 1 and their average sequence conservation (Figure 5a). Specifically, we generated Gly25→Val and Ala144→Val/Phe variants in an attempt to “shorten” pocket 1 so that acyl-CoAs more than 7 and 6 carbons long respectively do not “fit” if bound in a fully extended conformation. These variants were tested for activity using palmitoyl-CoA (C16-CoA) and their activities were compared against wild type (WT) Ma-WS/DGAT. The Gly25→Val mutation had an immense effect on the activity of the enzyme towards palmitoyl-CoA (1.0% of WT) while the Ala144→Val/Phe variants were still ~ 40–50% active (Table 1). Although, binding of palmitoyl-CoA was not expected in the case of the Ala144→Val/Phe mutants, these results suggest that palmitoyl-CoA may still bind in a different, less favorable conformation where the acyl chain is folded instead of fully extended. This binding mode may energetically be even less favorable in the case of the Gly25→Val mutant where pocket 1 is expected to be long enough to fit a 7-carbon long chain instead of 6. In addition to palmitoyl-CoA, we tested the activity of the Gly25→Val and Ala144→Phe variants towards

hexanoyl-CoA (C6-CoA). The Gly25→Val variant was 46.9% active compared to WT Ma-WS/DGAT while a 5.5-fold increase in activity was observed for the Ala144→Phe mutant (Table 1). This increased activity of the mutants towards the shorter acyl-CoA further supports the role of pocket 1 as the acyl chain-binding site.

To compare the substrate preference of wild type, Gly25→Val and Ala144→Phe Ma-WS/DGATs for acyl-CoA, we performed competition assays. Briefly, two different alcohols (hexanol and dodecanol) or two different acyl-CoAs (hexanoyl- and palmitoyl-CoA) were added in the reactions together at equal concentrations, and the wax esters formed (hexyl-caproate and dodecyl-caproate or hexyl-caproate and hexyl-palmitate respectively) were expressed as “percentage of total wax esters produced” by the enzyme. All three variants, wild type Ma-WS/DGAT and mutants, exhibited a clear preference towards the longer alcohol (dodecanol, C12-OH) and produced almost exclusively dodecyl-caproate (Figure 6a), showing that the mutations in pocket 1 did not alter the alcohol specificity of Ma-WS/DGAT. In contrast, both mutants showed a preference for the shorter acyl-CoA with Gly25→Val producing almost exclusively hexyl-caproate (~90% of total wax esters formed), and Ala144→Phe producing ~65% hexyl-caproate and ~35% hexyl-palmitate (Figure 6b). Although the Gly25→Val variant showed a clear preference for hexanoyl-CoA instead of palmitoyl-CoA, the Ala144→Phe was more efficient as it produced more of both products. Nonetheless, these results further demonstrate that the acyl chain of the acyl-CoA binds in pocket 1 as well as that Ma-WS/DGAT is amenable to structure-guided engineering towards the production of desired wax esters.

Catalytic Efficiency of Wild Type Ma-WS/DGAT and Mutants

The aforementioned end-point activity and competition assays showed that the Gly25→Val and Ala144→Phe mutants had a preference towards hexanoyl-CoA over palmitoyl-CoA in contrast to the wild type Ma-WS/DGAT (Figure 6). These assays also showed that the Ala144→Phe variant produced 6.5 times more hexyl-caproate than the wild type enzyme while Gly25→Val was less active than the wild type (Table 1). These assays, however, did not provide detailed information about the catalytic efficiency of the mutants in comparison with the wild type enzyme. For this reason, we pursued kinetic characterization of wild type (WT), Gly25→Val and Ala144→Phe Ma-WS/DGATs.

The challenges associated with the kinetic characterization of Ma-WS/DGAT have been previously discussed.²¹ Briefly, K_M values for long acyl-CoAs are too low to be determined because of the detection limit of the assays utilized. On the other hand, K_M values for short acyl-CoAs are too high and the low solubility of the substrates in the reaction buffer do not allow for saturation of the enzyme. Despite these challenges, we utilized a previously described method to monitor in real-time the release of CoA during the WS reaction, and obtained some more detailed kinetic data (Table 2, Figure S3).^{21–22, 56–57} Although dodecanol was a better substrate than hexanol for all three variants (Figure 6a), we used hexanol as the acyl acceptor in all kinetic experiments because of its higher solubility in the reaction buffer.

In the case of C16-CoA, we tested 35, 50, 100 and 200 μ M of substrate. All variants exhibited maximum rates at 50 μ M and were slightly inhibited at 100 and 200 μ M of C16-

CoA (Figure S3a, c, e). Since the assay was not sensitive enough to measure initial rates for concentrations lower than 30 μM , we could not obtain Michaelis-Menten curves and identify whether the maximum observed rates correspond to the k_{cat} of the enzymes or the C16-CoA had reached the critical micelle concentration (CMC) in the reaction conditions and its effective concentration was different above 50 μM . The reported CMCs for C16-CoA range from 8 μM to 550 μM based on the assay conditions (buffer, pH, temperature, salt concentration) as well as the method used to determine the CMC.^{64–66} Nonetheless, the maximum observed rates can be considered as apparent k_{cat} ($k_{\text{cat,app}}$) for comparison purposes. These data showed that the WT Ma-WS/DGAT consumed the C16-CoA ~ 1000 times faster than Gly25 $\rightarrow$ Val and ~ 34 times faster than Ala144 $\rightarrow$ Phe (Table 2).

In the case of C6-CoA, our data suggested K_{M} values higher than 2 mM for both WT and Gly25 $\rightarrow$ Val whereas the K_{M} of Ala144 $\rightarrow$ Phe was 165.6 μM (Table 2, Figure S3b, d, f). Both mutants were slower than the WT enzyme with the Gly25 $\rightarrow$ Val mutant being ~ 30 times slower at corresponding C6-CoA concentrations. Even though the Ala144 $\rightarrow$ Phe mutant was slower than the WT, its decreased K_{M} for C6-CoA made it a better catalyst at concentrations lower than ~ 700 μM (Figure S3b, d), which would explain the results of the end-point activity assay where 100 μM of C6-CoA was used (Table 1). The catalytic efficiency of Ala144 $\rightarrow$ Phe was ~ 2.76 times higher than that of the WT enzyme. Although the activity of the enzyme was significantly affected by the Gly25 $\rightarrow$ Val mutation, the mutant was about 10 times faster towards C6-CoA than C16-CoA at corresponding concentrations. This would justify the higher preference of Gly25 $\rightarrow$ Val for C6-CoA (Figure 6b) and the lower activity observed in the end-point assay (Table 1). Despite the lack of complete kinetic data for WT and mutants, our kinetic study combined with the competition assays further support that the acyl-chain of the acyl-CoA binds in pocket 1 and that Ma-WS/DGAT can be engineered to produce desired wax esters.

Role of Conserved Motif Residues

The proposed mechanism for WS/DGATs requires abstraction of a proton from an alcohol by a His residue to generate the reactive alkoxide (Scheme 1). However, the side chain of His is not sufficiently nucleophilic to abstract a proton from an alcohol ($\text{p}K_{\text{a}}$ of about 17). This raises the question how the catalytic His can abstract a hydroxyl proton from the acyl acceptor in the active site of WS/DGATs. In the crystal structure of Ma-WS/DGAT, His136 is positioned for catalysis in two ways. First, the other residues of the conserved motif HHXXXDG (HHSLVDG in Ma-WS/DGAT) form interactions that maintain the structural integrity of the loop and position His136 towards the enzyme cavity (Figure 1c–d). Second, the imidazole ring of His136 is oriented by π interactions with the aromatic ring of Phe11 (Figure 5b). There are no residues, however, within hydrogen bonding distance from His136. The only other highly conserved residue adjacent to the catalytic His is Asp8 (Figure 5b). This led us to the hypothesis that upon binding of the substrates, Asp8 might move closer to His136 and tune its $\text{p}K_{\text{a}}$, and similar movements of Asp-His dyads are observed in serine proteases.^{67–70} To test this hypothesis, we generated an Asp8 $\rightarrow$ Ala mutant and found that its activity was about half of the wild type (Table 1). However, Asp8 might be too far away from His136 (~ 6.0 Å) and a closer examination of the structure revealed that it is hydrogen bonded to the carbonyl oxygen of Ser137 (Figure 5b). This suggests that Asp8 may play a

structural role in proper positioning of His136 for catalysis by participating in maintaining the structural integrity of the loop formed by the conserved motif. In the structure of AtHCT, the catalytic His153 is in hydrogen bonding distance from Asn300. Comparatively, the structure of Ma-WS/DGAT is missing residues 296–308 which include the highly conserved Asn306 and semi-conserved Glu307 (Figure S7). It is likely that these residues may, in addition to environmental features of the active site, modulate the pK_a of His136.

CONCLUDING REMARKS

Our structural analysis of Ma-WS/DGAT reveals an acyltransferase-like fold that has been adapted to accommodate large hydrophobic substrates. The structural data in conjunction with our mutational analysis suggest that the acyl chain of the acyl-CoA binds in a pocket formed at the core of the N-terminal domain. Notably, introduction in this pocket of the highly hydrophobic Phe at position 144 resulted in an enzyme that was ~2.76 times more efficient towards a shorter acyl-CoA (C6-CoA) compared to the wild type. These results, in combination with previous studies,²⁶ further support the notion that the bacterial WS/DGATs are amenable to site-directed engineering. This work provides a framework for further engineering studies aimed at *in vivo* production of desired wax esters utilizing bacterial WS/DGATs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

(a) Ribbon diagram of the overall structure of Ma-WS/DGAT (one monomer shown). The N-terminal domain is shown in purple, the C-terminal domain in gray and the helical linker in yellow. (b) The cavity of Ma-WS/DGAT is shown in black, and the conserved motif HHHXXDGH (HHSLVDG in Ma-WS/DGAT) is colored in yellow. For description purposes, the cavity is divided into pocket 1 and pocket 2. (c) His135 points away from the enzyme cavity, and is in hydrogen bonding distance from Glu15, His136 and Ser137. All 4 residues are highly conserved. (d) Asp140 seems to play structural role forming interactions mainly with the peptide backbone. Distances shown are in Å.

Figure 2. Amino acid sequence conservation mapped onto the structure of Ma-WS/DGAT (as calculated by ConSurf).^{58–59} The rectangle indicates a “ring” of highly conserved residues around the active site.

Figure 3. Ribbon diagram and cavity of: (a) TRI3 trichothecene 15-O-acetyltransferase from *Fusarium sporotrichioides* (FsTRI3) (PDB ID 3FP0).⁶¹ The substrate (acetyl acceptor) is shown in sticks. (b) Hydroxycinnamoyl-CoA:shikimate hydroxycinnamoyltransferase from *Arabidopsis thaliana* (AtHCT) (PDB ID 5KJT).⁶³ The Coenzyme A (CoA) activated substrate (p-coumaroyl-CoA) is shown in sticks. (c) Ma-WS/DGAT (PDB ID: 6CHJ) with palmitoyl-CoA modeled based on the superposition with AtHCT. The catalytic histidine residues are shown in sticks. (d, e, f) Comparison of the residues that line and constrict the pocket at the core of the N-terminal domains of FsTRI3, AtHCT and Ma-WS/DGAT respectively.

Figure 4.

Alcohol binding pocket (pocket 2) of Ma-WS/DGAT colored based on amino acid conservation as calculated by ConSurf.^{58–59} The side chains of the pocket lining residues are shown in sticks. Leu356, Ala360 and Met405, which have been shown to alter the enzyme's alcohol specificity towards shorter chain lengths, are designated with red font.^{26, 62} Residues that are distal to the catalytic His136 and have low to average conservation are good candidates for engineering efforts that aim to alter alcohol specificity.

Figure 5. Ribbon diagram and cavity of Ma-WS/DGAT colored based on amino acid conservation as calculated by ConSurf.^{58–59} (a) Gly25 and Ala144 are located on strand β1 and helix α4 respectively lining the pocket where the acyl chain binds. (b) Positioning of His136 for catalysis by the conserved motif loop HHSLVGD (shown in sticks) and Phe11. Asp8 is also involved in maintaining the structural integrity of the loop. Distances shown are in Å.

Figure 6. Percentage of total wax esters produced by Ma-WS/DGAT wild type (WT) and mutants when (a) hexanoyl-CoA (C6-CoA) and both hexanol (C6-OH) and dodecanol (C12-OH) were added in the reactions, and (b) hexanol (C6-OH) and both hexanoyl- (C6-CoA) and palmitoyl-CoA (C16-CoA) were added in the reactions. Results are means $\pm$ SEM of duplicate experiments.

Scheme 1.

Proposed mechanism of bacterial WS/DGAT. A catalytic histidine of the conserved motif HHXXXDG abstracts a proton from the hydroxyl moiety of the substrate generating an alkoxide ion. Attack of the alkoxide onto the carbonyl carbon of the acyl-CoA thioester produces a tetrahedral intermediate, which subsequently collapses to yield the acylated alcohol and free CoA. Abbreviations: DAG for diacylglycerol and CoA for Coenzyme A.

Table 1.

Activity of wild type Ma-WS/DGAT and mutants. The activity of the mutants was expressed as “percentage of wax ester produced” by each variant in respect to the wild type reaction (considered as 100%). Results are means of duplicate experiments. (ND = Not Determined)

Ma-WS/DGAT	hexyl-palmitate (%)	hexyl-caproate (%)
WT	100	100
H136N	1.5	ND
D140A	49.6	ND
D8A	24.1	ND
G25V	1.0	46.9
A144V	43.3	ND
A144F	53	654.6

Table 2.

Kinetic data of wild type, G25V and A144F Ma-WS/DGATs. Due to limitations of the assay and the nature of the substrates, a Michaelis - Menten curve could be obtained only for the A144F mutant. The observed rates for all variants are shown in Figure S3. Results are means $\pm$ SEM of duplicate experiments.

Ma-WS/DGAT	C16-CoA		C6-CoA	
	$k_{\text{cat,app}}$ (s^{-1})	$k_{\text{cat,app}}$ (s^{-1})	K_M (μM)	k_{cat}/K_M ($\text{s}^{-1}\text{M}^{-1}$)
WT	6.53 ± 0.49	> 3	> 1900	1860 ± 70
G25V	0.007 ± 0.001	> 0.09	> 2400	30.9 ± 0.5
A144F	0.19 ± 0.01	0.85 ± 0.03	165.6 ± 15.1	5133 ± 264