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Structural Control of Chemoselectivity, Stereoselectivity, and Substrate Specificity in Membrane-Bound Fatty Acid Acetylenases and Desaturases[†]

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ABSTRACT: The FAD2-like desaturases comprise a group of membrane-bound oxygenases involved in the modification of fatty acyl groups in plants and fungi. This group includes typical oleate desaturases which introduce a Δ12 *cis* double bond and more unusual enzymes such as Crep1, an acetylenase from the plant *Crepis alpina*, which introduces a triple bond in linoleate at the Δ12 position. In this study, the structure−function relationship between FAD2-like acetylenases and desaturases was examined through site-directed mutagenesis and heterologous expression. Eleven amino acid positions were identified that show complete evolutionary conservation within acetylenases or desaturases but have different amino acids in the other class of enzyme. Point mutants in *Crep1* were constructed and expressed in yeast to test the role in fatty acid modification of the amino acids at the 11 positions. Results indicate the importance of five amino acid positions within Crep1 with regard to desaturase and acetylenase chemoselectivity, stereoselectivity, and substrate recognition. For example, relative to wild-type Crep1, the Y150F, F259L, and H266Q mutations all favored desaturation over acetylenation. The data indicate that small changes in primary sequence, particularly in the vicinity of the active site, can have profound changes on chemoselectivity and other aspects of the function of membrane-bound desaturase-like enzymes.

The FAD2[1]-like desaturases comprise a group of membrane-bound oxygenases involved in the modification of fatty acyl groups in plants and fungi. The archetypal plant FAD2 catalyzes the introduction of a *cis* double bond at the Δ12 position of oleoyl-phosphatidylcholine in the endoplasmic reticulum (*1*) (Figure 1). This requires molecular oxygen and reducing equivalents from cytochrome b_5 (*2*).

Interestingly, the FAD2-like enzyme group includes a number of variants which catalyze reactions other than the usual Δ12 *cis* desaturation. These reactions include hydroxylation, epoxidation, conjugated double bond formation, and triple bond formation (*3*). The acetylenase Crep1, from *Crepis alpina*, is the first of a number of FAD2-like acetylenases identified in the plant families Asteraceae, Apiaceae, and Araliaceae (*4*). Crep1 catalyzes the formation of a triple bond at the 12,13 position of linoleate (18:2-9c,12c) to give crepenynate (18:1-9c,12a) (Figure 1). Crep1 has also been reported to catalyze both *cis* and *trans* desaturation of oleate (*5*) (Figure 1).

In general, the membrane-bound desaturases are particularly difficult to work with *in vitro*. Much of what is known about the structure and function of these enzymes has been derived from their primary structure and *in vivo* activities in yeast. By analogy to soluble fatty acid desaturases, the presence of conserved His boxes of the formulas $HX_{3-4}H$, $HX_{2-3}HH$, and $(H/Q)X_{2-3}HH$ has led to the suggestion that these include the ligands for a diiron cluster at the active site of the enzyme (*6*) (Figure 2). Amino acid residues that are proposed to contribute to activity tend to cluster around these His boxes (*7*−*10*) while hydropathy plots suggest four conserved transmembrane domains (*11*) (Figure 2).

The similarity between plant FAD2s and their variants is often quite high. Crep1, for example, shares 56% amino acid sequence identity with the *Arabidopsis thaliana* FAD2. This high degree of similarity suggests the feasibility of probing structure−function relationships in this group of enzymes through site-directed mutagenesis. Such an approach has been used successfully in probing the relationship between Δ12 desaturation and 12-hydroxylation (*7*).

In this study, the structure−function relationships within FAD2-like enzymes were investigated with a focus on acetylenation and desaturation. A comparison of FAD2 desaturase and acetylenase sequences was used to design mutants of Crep1 which were then analyzed by heterologous expression in yeast. The results reveal interesting findings regarding the structural determinants of chemoselectivity, stereoselectivity, and substrate specificity in these enzymes.

EXPERIMENTAL PROCEDURES

Sequence Alignment. Thirty-eight FAD2-like sequences from 17 plant families and 1 algal family were collected; 13 of the sequences have been confirmed previously to represent oleate desaturases by heterologous expression in yeast, soybean, or pig.

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[1]Abbreviations: Crep1, Δ12 acetylenase from *Crepis alpina*; FAD2, extraplastidial oleate desaturase; FAME, fatty acid methyl ester; FID, flame ionization detection; GC, gas chromatography; GC-MS, gas chromatography−mass spectrometry; MS, mass spectrometry; ORF, open reading frame; SD-URA, synthetic dextrose medium supplemented with amino acids (excluding uracil); SE, standard error.

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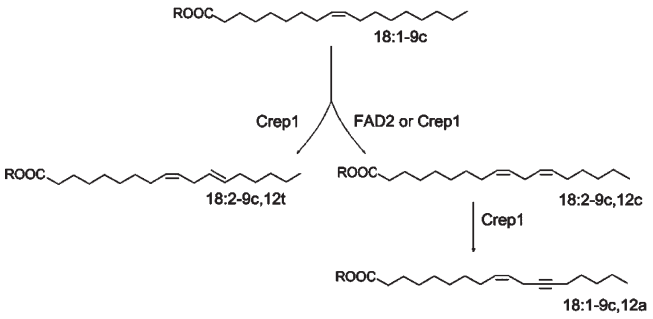

FIGURE 1: The FAD2 and Crep1 reactions indicating the multifunctional activity of Crep1.

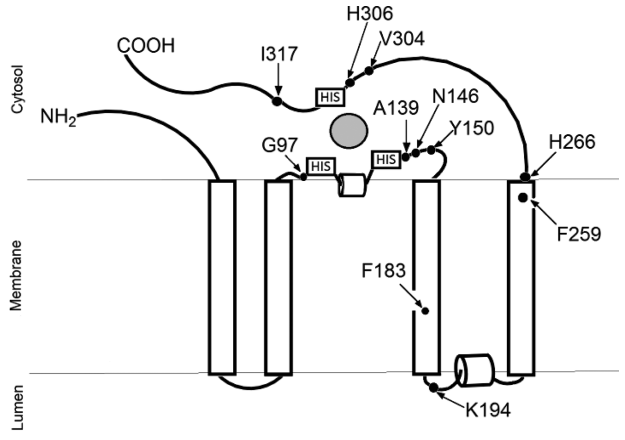

FIGURE 2: A topological model for membrane-bound fatty acid desaturases (*11*). A putative diiron center (gray circle) and three His boxes are shown. Black circles indicate amino acid positions which are completely conserved in either acetylenases or FAD2's but different in the other enzyme type, as examined in this study. Amino acid positions are numbered according to the Crep1 sequence.

The remaining 25 sequences showed a 71−99% amino acid sequence identity to *Arabidopsis* FAD2. Similarly, 5 confirmed and 7 putative acetylenase sequences were collected. The latter 7 showed a 52−94% sequence identity with the *Petroselinum crispum* acetylenase sequence. Amino acid sequences were aligned using CLUSTALW (*12*) using default parameters (see Supporting Information Figure S1).

Site-Directed Mutagenesis of Crep1. The Crep1 ORF was previously subcloned into the pVT100U vector (*4*, *13*) to create pVTCrep1. Both plasmids were kindly provided by Dr. Sten Stymne (Swedish University of Agricultural Sciences) and were used throughout this study as a wild type (pVTCrep1) and negative (pVT100U) control. Based on the results of the sequence alignment (see above), point mutations corresponding to selected amino acid positions within the Crep1 ORF of pVTCrep1 were generated using a QuikChange II site-directed mutagenesis kit (Stratagene, La Jolla, CA) following the manufacturer's instructions. The sequence of the resulting plasmids was determined using an ABI Prism Big Dye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA) with a 3730x1 DNA analyzer (Applied Biosystems) at the National Research Council of Canada Plant Biotechnology Institute (Saskatoon, Saskatchewan). The oligonucleotide primers utilized in this study are indicated in Supporting Information Table S1. Primers used to introduce point mutations into pVTCrep1 were designed using PrimerX software (http://www.bioinformatics.org/primerx/index.htm).

Transformation and Growth of Yeast. Plasmid constructs were individually transformed into *Saccharomyces cerevisiae* strain InvSc1 (*MATa his3D1 leu2 trp1−289 ura3−52*) using the S.c. EasyComp transformation kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Starter cultures of transformed yeast were grown for 48 h at 28 °C in 1 mL of SD-URA (BIO 101, Inc., Vista, CA). A 100 μL aliquot of starter culture was then used to inoculate 10 mL of SD-URA in 50 mL Erlenmeyer flasks. In some cases, linoleic acid (18:2-9c,12c; Nu-Chek Prep, Inc., Elysian, MN) was added to cultures as 100 μg/mL free acid in 0.1% (v/v) tergitol (Sigma, St. Louis, MO). Fatty acid accumulation was measured in three independent experiments that were performed with triplicate cultures. Cultures were grown for 3 days at 20 °C followed by another 3 days at 15 °C on a platform shaker at 200 rpm (*14*).

Gas Chromatography and Mass Spectrometry. Cultures grown in the presence of 18:2-9c,12c were centrifuged at 3000 rpm for 5 min and washed once with 1 mL of 1% (v/v) tergitol and twice with 5 mL of H_2O to remove extracellular 18:2-9c,12c. Cultures grown in the absence of 18:2-9c,12c were centrifuged and washed once with 5 mL of H_2O. One hundred microliters of 17:0 (100 μg/mL in methanol; Nu-Chek Prep, Inc.) was added to each sample to act as an internal standard. Sample preparation involved the saponification of fatty acids using 1 mL of 10% KOH (w/v) in methanol and heating the reaction mixture in a sealed glass tube with a Teflon-lined lid to 80 °C for 1 h. Mixtures were then extracted with 1 mL of hexane, followed by acidification with 500 μL of 50% (v/v) acetic acid. Fatty acids were then extracted using 2 mL of hexane and concentrated under a nitrogen stream. The residue was methylated by heating to 60 °C in the presence of 1 mL of 1% (v/v) sulfuric acid in dry methanol for 1 h. The resulting FAMEs were extracted from the mixture using 2 mL of hexane after addition of 1 mL of H_2O. The FAMEs were then concentrated under a nitrogen stream to dryness followed by the addition of 200 μL of ethyl acetate.

The methyl esters were analyzed by GC-MS using an Agilent 6890N GC (Santa Clara, CA) with a DB-23 column (30 m × 0.25 mm i.d.; J&W Scientific, Folsom, CA), 25:1 split flow injector at 225 °C, and a column oven temperature program of 160 °C for 1 min and then 4 °C/min to 240 °C connected to an Agilent 5973N mass selective detector in electron impact mode under standard conditions (70 eV). GC analysis was done using an Agilent 6890 GC with a similar column and the oven under the same conditions connected to a flame ionization detector. FAMEs were quantified by peak area integration using Chemstation software (Agilent, Palo Alto, CA) and assuming equivalent FID response factors on a weight basis. Identification of fatty acid methyl esters is described in the Supporting Information (Figures S2 and S3).

RESULTS

Crep1 Mutants. Typically, evolutionary conservation of amino acids at particular positions in a protein sequence is taken to suggest the importance of those amino acids in its function (*15*). Similarly, conserved differences in amino acid conservation may suggest their importance in different functions in homologous enzymes. With this in mind, and with a view toward understanding the determinants of desaturase and acetylenase function in FAD2 homologues, amino acid sequences of plant oleate desaturases and acetylenases were collected, aligned, and examined for conserved differences. The resulting alignment contained 38 putative or experimentally confirmed oleate desaturases (FAD2s) representing 17 plant families and 1 algal family and 12 putative or experimentally confirmed plant acetylenases from

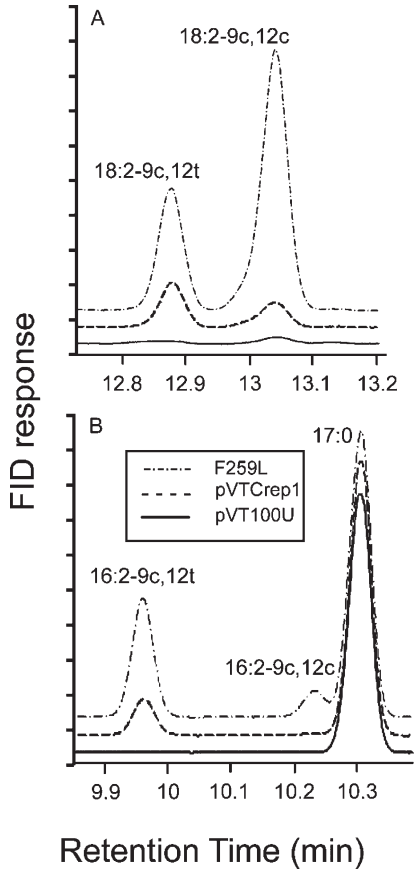

FIGURE 3: Desaturation products of Crep1. Gas chromatograms show peaks corresponding to (A) 18- and (B) 16-carbon products of yeast grown in the absence of 18:2-9c,12c after transformation with pVT100U (empty vector), pVTCrep1 (wild-type Crep1), and the plasmid corresponding to the mutant F259L.

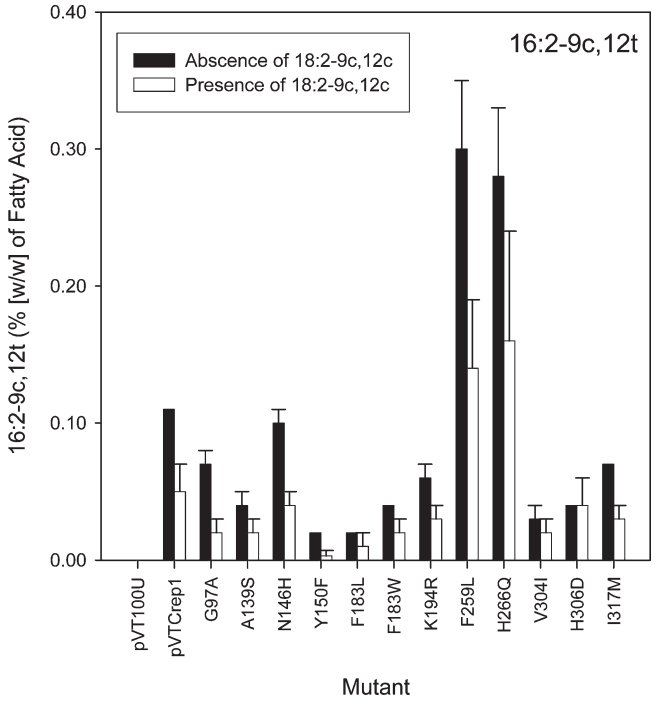

FIGURE 4: Accumulation of 16:2-9c,12t in wild-type (pVTCrep1) and mutants of Crep1. pVT100U (empty vector) was used as a negative control. Cultures were grown for 6 days in the absence (black bars) or presence (white bars) of exogenous 18:2-9c,12c and analyzed by GC. Values represent means ± SE (n = 3). See also Tables S2 and S3.

the Asteraceae, Apiaceae, and Araliaceae (see Supporting Information Figure S1).

When only FAD2 sequences for which there was experimental confirmation of function were considered, inspection of the alignment revealed 14 amino acid positions which had the following two properties: (a) complete conservation of amino acid identity in one of the two groups of enzymes and (b) no occurrence of the conserved amino acid in the other groups of enzymes. If all 38 sequences were considered, the number of such "conserved difference" positions was reduced to 11 (see Supporting Information Figure S1). Despite the tentative functional assignment of some of the sequences in the alignment, a decision was made to focus on the importance of the 11 positions in determining enzyme function.

The positions of the 11 conserved differences determined from the alignment are shown in Figure 2, overlaid on a topological model established for membrane-bound desaturases (*11*). It is notable that all but 2 of the positions are predicted to be on the cytosolic side of the endoplasmic reticulum and 7 out of the 11 positions are within 12 amino acids of one of three His boxes which are thought to be involved in binding a diiron cluster at the active site of these enzymes. Experiments were designed to test the effect of mutation at the 11 amino acid positions in a Crep1 background. In other words, individual Crep1 mutants were constructed such that, for amino acid positions 97, 139, 146, 150, 183, 194, 259, 266, 304, 306, and 317, the mutant gene products contained amino acids corresponding to the *Crepis palaestina* FAD2 sequence. A twelfth mutant designated F183L, an incidental product of the mutagenesis work, was also included in this study.

Crep1 Products. In order to test the function of mutants of Crep1, yeast was transformed with plasmids designed to express the mutants. Transformants corresponding to the empty vector (pVT100U) and wild-type Crep1 (pVTCrep1) were used for comparison. Yeast cultures were grown for 6 days in the absence or presence of 18:2-9c,12c. In the absence of 18:2-9c,12c, the ability of Crep1 mutants to desaturate endogenous monoenoic acids could be tested (Supporting Information Table S2). In the presence of 18:2-9c,12c, acetylenase activity could be tested (Supporting Information Table S3). For all yeast cultures grown in the absence of exogenous 18:2-9c,12c, the corresponding gas chromatograms of fatty acid methyl esters characteristically showed four major peaks corresponding to 16:0 [19−20% (w/w)], 16:1-9c [45−46% (w/w)], 18:0 [6−7% (w/w)], and 18:1-9c [28−29% (w/w)] (Supporting Information Table S2; data not shown). Of course, in the presence of 18:2-9c,12c, an additional major peak corresponding to 18:2-9c,12c appeared (Supporting Information Table S3; data not shown). The products of Crep1 and its mutants did not exceed 1% (w/w) of total fatty acids and therefore had little effect on the overall appearance of the gas chromatograms (Supporting Information Tables S2 and S3; data not shown).

The desaturase activity of wild-type Crep1 is illustrated in Figure 3. As reported previously (*5*) both *cis* and *trans* desaturation of endogenous 18:1-9c was detected in a ratio of about 1:3 (*cis:trans*, Figure 3A, dashed line). In addition to this, a previously unreported desaturation of 16:1-9c was evident. In this case, the product was apparently all in the 12t configuration (Figure 3B, dashed line). The product was tentatively identified as 16:2-9c,12t based on GC-MS and the assumption of Δ12 desaturation (see Supporting Information Figures S2 and S3).

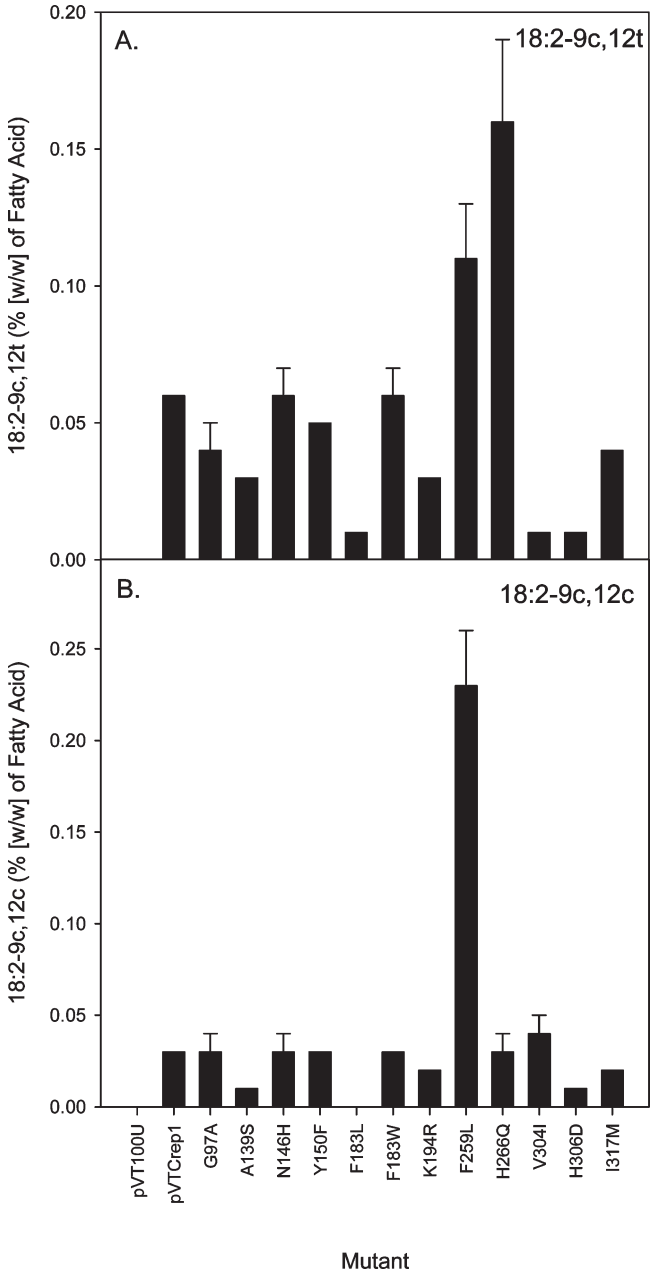

FIGURE 5: Accumulation of (A) 18:2-9c,12t and (B) 18:2-9c,12c in wild-type (pVTCrep1) and mutants of Crep1. pVT100U (empty vector) was used as a negative control. Cultures were grown for 6 days in the absence of exogenous 18:2-9c,12c and analyzed by GC. Values represent means ± SE (n = 3). See also Supporting Information Table S2.

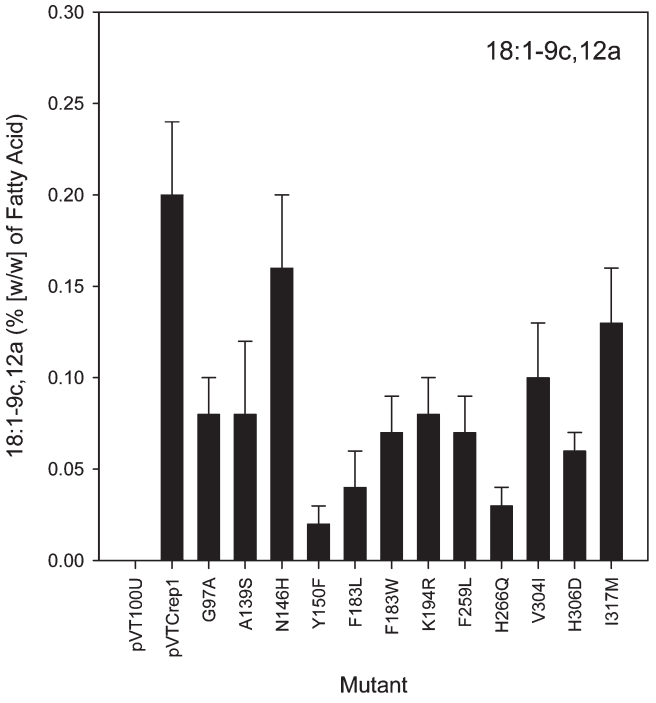

FIGURE 6: Accumulation of 18:1-9c,12a (crepenynic acid) in wild-type (pVTCrep1) and mutants of Crep1. pVT100U (empty vector) was used as a negative control. Cultures were grown for 6 days in the presence of exogenous 18:2-9c,12c and analyzed by GC. Values represent means ± SE (n = 3). See also Supporting Information Table S3.

In Vivo Characterization of Crep1 Mutants. The isomers of 16:2 were quantified by GC for yeast cultures grown in both the presence and absence of 18:2-9c,12c in the growth medium (Figure 4, Supporting Information Tables S2 and S3). In the absence of exogenous 18:2-9c,12c, cultures expressing wild-type Crep1 accumulated 16:2-9c,12t to levels of 0.11% (w/w) (Figure 4, Supporting Information Table S2) but did not accumulate 16:2-9c,12c isomer in detectable amounts (Supporting Information Table S2). While the presence of 18:2-9c,12c in the medium affected 16:2-9c,12t accumulation as a percentage of total fatty acids (Figure 4; Supporting Information Table S3), results with and without 18:2-9c,12c were highly correlated (Figure 4, Supporting Information Tables S2 and S3). The largest producers of 16:2-9c,12t were seen in cultures harboring plasmids corresponding to F259L and H266Q, with accumulations which were 2.5−3.2-fold higher than wild-type Crep1 (Figure 4, Supporting Information Tables S2 and S3). Other yeast strains harboring mutant Crep1 constructs produced less 16:2-9c,12t isomer than yeast cultures expressing Crep1. The smallest amount of 16:2-9c,12t among these cultures was detected in yeast strains corresponding to Y150F and F183L where accumulation was reduced by 80−94% in comparison to wild-type Crep1 (Figure 4, Supporting Information Tables S2 and S3).

Curiously, of all cultures analyzed, only two transformed strains accumulated a fatty acid identified as 16:2-9c,12c as detected by GC-MS (Figure 3B, dashed/dotted line, and Supporting Information Tables S2 and S3). Yeast strains corresponding to the F259L and V304I mutants were found to contain 16:2-9c,12c at 0.06 ± 0.01% (w/w) and 0.02 ± 0.003% (w/w), respectively, in the absence of 18:2-9c,12c (Supporting Information Table S2). In the presence of 18:2-9c,12c, the two mutants also produced small amounts of 16:2-9c,12c (Supporting Information Table S3). It is interesting to note that 16:2-9c,12c has been reported as a product of *Arabidopsis* FAD2 and other oleate desaturases (*16*).

The accumulation of linoleate isomers in yeast cultures grown in the absence of exogenous 18:2-9c,12c is shown in Figure 5. As with the *trans* desaturation product of 16:1-9c, the accumulation of 18:2-9c,12t was affected the most, relative to wild-type Crep1, in the cultures corresponding to the mutants F183L, V304I, and H306D, all of which showed 83% decrease, while F259L and H266Q demonstrated 1.8- and 2.7-fold increases, respectively (Figure 5A, Supporting Information Table S2). Upon examining the same mutants for the accumulation of 18:2-9c,12c, V304I increased slightly in comparison to wild-type Crep1 while H306D decreased. For F183L, 18:2-9c,12c production was essentially eliminated. While there was no effect in the

Table 1: Effects of Mutations in Crep1 on Chemoselectivity, Stereoselectivity, and Substrate Specificity[a]

mutant	*cis* desaturation: acetylenation	fold change	*trans* desaturation: acetylenation	fold change	total *cis*: total *trans*	fold change	total 16:2/total 18:2	fold change
Crep1	0.14 ± 0.03[b]		0.80 ± 0.17		0.18 ± 0.01		1.26 ± 0.07	
G97A	0.33 ± 0.10	2.4	1.47 ± 0.35	1.8	0.22 ± 0.06	1.2	1.05 ± 0.25	0.8
A139S	0.16 ± 0.09	1.1	0.85 ± 0.41	1.1	0.19 ± 0.05	1.1	1.11 ± 0.22	0.9
N146H	0.19 ± 0.06	1.4	1.01 ± 0.28	1.3	0.19 ± 0.05	1.1	1.17 ± 0.23	0.9
Y150F	1.52 ± 0.42	10.9	3.73 ± 0.96	4.7	0.41 ± 0.05	2.3	0.19 ± 0.02	0.2
F183L	0[c]	−[d]	0.77 ± 0.45	1.0	0[c]	−[d]	2.53 ± 0.76	2.0
F183W	0.39 ± 0.14	2.8	1.33 ± 0.47	1.7	0.29 ± 0.04	1.6	0.42 ± 0.06	0.3
K194R	0.22 ± 0.06	1.6	1.21 ± 0.33	1.5	0.18 ± 0.03	1.0	1.26 ± 0.14	1.0
F259L	3.89 ± 0.99	27.7	5.58 ± 1.51	7.0	0.70 ± 0.12	3.9	1.07 ± 0.19	0.8
H266Q	0.89 ± 0.35	6.4	15.6 ± 5.57	19.5	0.06 ± 0.01	0.3	1.55 ± 0.38	1.2
V304I	0.57 ± 0.16	4.1	0.38 ± 0.11	0.5	1.48 ± 0.28	8.2	1.11 ± 0.20	0.9
H306D	0.18 ± 0.03	1.3	0.85 ± 0.17	1.1	0.21 ± 0.02	1.2	2.05 ± 0.39	1.6
I317M	0.14 ± 0.03	1.0	0.78 ± 0.16	1.0	0.18 ± 0.01	1.0	1.18 ± 0.06	0.9

[a]Estimated as *cis* desaturation:acetylenation, *trans* desaturation:acetylenation, *cis:trans* products, and 16:2/18:2, respectively. Fold changes are relative to Crep1. [b]Values represent means ± SE (n = 3). [c]*cis* desaturation not detected. [d]Not calculated.

H266Q mutant, the F259L stood out with a 7.7-fold increase relative to wild-type Crep1 (Figure 5B, Supporting Information Table S2). This mirrors its distinctive *cis* desaturation of 16:1-9c.

Yeast cultures that expressed Crep1 accumulated crepenynate (18:1-9c,12a) to levels of 0.20 ± 0.04% (w/w) when grown in the presence of 18:2-9c,12c (Figure 6, Supporting Information Table S3). The desaturation product 16:2-9c,12t was also detected in these cultures (Supporting Information Table S3). Relative to the wild-type Crep1, the mutants demonstrated a 20−90% drop in crepenynate levels (Figure 6, Supporting Information Table S3). Yeast strains harboring the mutants Y150F (90% reduction) and H266Q (85% reduction) were the most significantly affected (Figure 6, Supporting Information Table S3).

Chemoselectivity, Stereoselectivity, and Substrate Specificity of Crep1 Mutants. While comparing the data from fatty acid accumulation suggests amino acid positions which are important for Crep1 function, on their own, they are limited by possible confounding factors such as differential expression (protein level) of various mutants in yeast. It is possible to circumvent such problems by relying to a large extent on ratios of fatty acid accumulation. In this way it is possible to tease out the effect of mutations on the chemoselectivity, stereoselectivity, and substrate selectivity of Crep1.

In this particular case, chemoselectivity refers to the relative propensity of Crep1 and related enzymes to perform either desaturation or acetylenation. It is interesting to examine the effect of mutations in Crep1 on these reactions. To this end, the ratios of *cis* desaturation to acetylenation were calculated by considering the sum of 16:2-9c,12c and 18:2-9c,12c product accumulation in cultures not containing 18:2-9c,12c as a function of 18:1-9c,12a product accumulation in cultures that had been supplied with 18:2-9c,12c for the various Crep1 constructs (Table 1). The wild-type ratio was 0.14, and the most dramatic increases from this value are evident for the mutants Y150F (10.9-fold increase), F259L (27.7-fold increase), and H266Q (6.4-fold increase) (Table 1). This suggests that these amino acid positions are important in determining chemoselectivity, mutations in the direction of FAD2-like sequence encouraging desaturase activity relative to acetylenase activity. On the other hand, *cis* desaturation was not detectable in the F183L mutant (Table 1).

The ratio of *trans* desaturation to acetylenation was considered by evaluating the accumulation of 16:2-9c,12t and 18:2-9c,12t in cultures grown in the absence of 18:2-9c,12c alongside the accumulation of 18:1-9c,12a product in cultures that had been supplied with 18:2-9c,12c. The most striking increases were also seen in Y150F (4.7-fold increase), F259L (7-fold increase), and H266Q (19.5-fold increase), again suggesting the importance of these residues in determining chemoselectivity (Table 1).

To determine if point mutations affect the geometry of desaturation products, the total *cis* isomer accumulation (16- and 18-carbon products) was compared to the total *trans* isomer formation in transgenic yeast cultures grown in the absence of exogenous 18:2-9c,12c (Table 1). The two mutant constructs showing the greatest increase in relative production of *cis* (versus *trans*) isomers when compared to wild-type Crep1 were F259L, which shows a 3.9-fold increase, and V304I, which had a 8.2-fold increase (Table 1). In this way, these mutants resemble FAD2's which do not catalyze *trans* desaturation to any detectable extent. On the other hand, the F183L (elimination of *cis* desaturation) and H266Q (3-fold decrease) mutants appeared to favor *trans* desaturation as compared to wild-type Crep1.

To determine if Crep1 mutants showed a change in preference for 16:1-9c versus 18:1-9c as a substrate for desaturation, the 16:2/18:2 product ratios were calculated and compared (Table 1). The total amounts of 16:2 geometric isomers were compared to the total 18:2 isomer accumulation for each yeast strain grown in the absence of exogenous 18:2-9c,12c. The mutants F183W and Y150F showed very significant increases (3- and 6.6-fold, respectively) in their preference for 18:1-9c as a substrate while F183L and H306D demonstrated 2- and 1.6-fold increases, respectively, in their preference for 16:1-9c as a substrate. Other mutations did not appear to substantially affect substrate selectivity (Table 1).

DISCUSSION

In this study, we have compared different aspects of activity, specifically, the chemoselectivity, stereoselectivity, and substrate selectivity of Crep1 and the effect of mutations at conserved sites on activity. Table 2 emphasizes the mutations which contribute to major qualitative changes in Crep1 function. Based on this, it is possible to make some interesting observations about the structure−function relationships in the membrane-bound desaturase-like group.

Previously, researchers have reported the effects of mutants on the function of enzymes related to membrane-bound fatty acid desaturases. This includes an investigation of chemoselectivity in

Table 2: Summary Based on Table 1 of the Major Qualitative Effects of Mutations in Crep1 on Chemoselectivity, Stereoselectivity, and Substrate Specificity

mutant	desaturation: acetylenation	*cis* desaturation:*trans* desaturation	total 16:2/total 18:2
Y150F	↑	↑	↓
F183L	↓	↓	↑
F183W	↑	↑	↓
F259L	↑	↑	–[a]
H266Q	↑	↓	–
V304I	–	↑	–

[a]Minimal change observed.

oleate 12-hydroxylase, regioselectivity in FAD2-like fungal desaturases, and substrate specificity in a fungal Δ6 desaturase (*7*, *14*–*17*). It is interesting to note that all of the relevant mutations which showed important changes in hydroxylase activity cluster near the His boxes in these enzymes. It has been proposed that these His boxes bind iron at the active site and that the mutations are therefore clustered around the active site of the enzyme. Similar to previous work, most of the mutations considered in this study are located near a conserved His box, although some of the amino acid changes affecting Crep1 activity, e.g., at positions 183, 259, and 266, are somewhat more distant, in a linear sequence sense.

In terms of chemoselectivity of Crep1, we have identified positions 150, 183, 259, and 266 as being important in determining the desaturation/acetylenation ratio. The first of these is predicted to be near the second His box, and the corresponding Y150F mutant represents a structural difference of only one oxygen atom. The region in the vicinity of position 150 may be important for desaturase function in general. For example, Meesapyodsuk and co-workers (*9*) found a major change in ω3 versus Δ12 regioselectivity of the desaturase CpDes12 from the fungus *Claviceps purpurea* upon mutation of the position (206) homologous to 149 in Crep1.

Position 183 is predicted to be near the middle of the third transmembrane domain, and positions 259 and 266 are probably near the cytosolic end of the fourth transmembrane domain. This is in rather stark contrast to the positions which affect the hydroxylation/desaturation ratio in the castor oleate 12-hydroxylase (95, 97, 315, and 317, Crep1 numbering) (*7*) which are near the first and third His boxes in terms of linear sequences. Thus, the structural features which control acetylenation appear to be quite distinct from those controlling hydroxylation.

Desaturation to give *trans* double bonds co-occurs with acetylenase activity in Crep1. It is possible to argue that similar spatial accommodation of the substrates and products for the *trans*-desaturation and acetylenation reactions may explain this observation. *trans* desaturation likely requires the binding of oleate in an antiperiplanar conformation and accommodation of the relatively "linear" 18:2-9c,12t product. Similarly, although the substrate for acetylenation is *cis* at the active site, the product is linear from C11 to C14. It is notable, therefore, that effects on acetylenation and *trans* desaturation are correlated for positions 150, 183, and 259. The notable exception is the H266Q mutation. Relative to the wild type, acetylenation is much reduced in comparison to *trans* desaturation in this mutant. In fact, H266Q represents the "accidental engineering" of an enzyme with primarily oleate *trans*-Δ12 desaturase activity. This suggests that the structural control of the two activities is independent to some extent. Indeed, this is supported by the occurrence of the oleate *trans*-Δ12 desaturase of *Dimorphotheca sinuata* (DsFAD2-1), which has no reported acetylenase activity and is generally more oleate *cis*-Δ12 desaturase-like at positions homologous to 150, 183, 250, 266, and 304 in Crep1 (*18*).

Certain mutations in Crep1 provide some intriguing hints about desaturase structure. The important effects of mutants at positions 259 and 266 suggest that these may be close to the active site. Position 183 appears to be important in selectivity for 16- versus 18-carbon fatty acids. Current topological models would predict that this position corresponds to the middle of the third transmembrane domain, which may be relatively distant from the active site. It is tempting to speculate that position 183 is involved in substrate binding at a location removed from the active site, perhaps near the methyl end of the substrate.

In summary, these data indicate that a small change in primary sequence, particularly in the vicinity of the active site, can have profound changes on chemoselectivity and other aspects of the function of membrane-bound desaturase-like enzymes.

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SUPPORTING INFORMATION AVAILABLE

The acetylenase/desaturase amino acid sequence alignment (Figure S1), oligonucleotide primers (Table S1), identification of fatty acids (including Figures S2 and S3), fatty acid content of yeast cultures grown in the absence of 18:2-9c,12c (Table S2), and fatty acid content of yeast cultures grown in the presence of 18:2-9c,12c (Table S3). This material is available free of charge via the Internet at http://pubs.acs.org.

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