THE JOURNAL OF BIOLOGICAL CHEMISTRY Vol. 277, No. 18, Issue of May 3, pp. 15613–15620, 2002
Printed in U.S.A.

Desaturation and Hydroxylation

RESIDUES 148 AND 324 OF *ARABIDOPSIS* FAD2, IN ADDITION TO SUBSTRATE CHAIN LENGTH, EXERT A MAJOR INFLUENCE IN PARTITIONING OF CATALYTIC SPECIFICITY*

Received for publication, January 9, 2002, and in revised form, February 14, 2002
Published, JBC Papers in Press, February 25, 2002, DOI 10.1074/jbc.M200231200

John A. Broadwater, Edward Whittle, and John Shanklin‡

From the Biology Department, Brookhaven National Laboratory, Upton, New York 11973

Exchanging the identity of amino acids at four key locations within the *Arabidopsis thaliana* oleate desaturase (FAD2) and the *Lesquerella fendleri* hydroxylase/desaturase (LFAH) was shown to influence partitioning between desaturation and hydroxylation (Broun, P., Shanklin, J., Whittle, E., and Somerville, C. (1998) *Science* 282, 1315–1317). We report that four analogous substitutions in the FAD2 sequence by their equivalents from the castor oleate hydroxylase result in hydroxy fatty acid accumulation in *A. thaliana* to the same levels as for the wild-type castor hydroxylase. We also describe the relative contribution of these substitutions, both individually and in combination, by analyzing the products resulting from their expression in *A. thaliana* and/or *Saccharomyces cerevisiae*. Yeast expression showed that M324V, a change reachable by a single point mutation, altered the product distribution ~49-fold, and that residue 148 is also a predominant determinant of reaction outcome. Comparison of residues at position 148 of FAD2, LFAH, and the *Ricinus* oleate hydroxylase prompted us to rationally engineer LFAH-N149I, a variant with ~1.9-fold increase in hydroxylation specificity compared with that of wild-type LFAH. Control experiments showed that the wild-type *Arabidopsis thaliana* FAD2 desaturase has inherent, low level, hydroxylation activity. Further, fatty acid desaturases from different kingdoms and with different regiospecificities exhibit similar intrinsic hydroxylase activity, underscoring fundamental mechanistic similarities between desaturation and hydroxylation. For LFAH mutants the hydroxylation:desaturation ratio is 5–9-fold higher for 18-carbon *versus* 16-carbon substrates, supporting our hypothesis that substrate positioning in the active site plays a key role in the partitioning of catalytic specificity.

Hydroxy fatty acids are unusual fatty acids that are incorporated into seed triacylglycerols in several species of plants, the best characterized being *Ricinus communis* (castor) and *Lesquerella fendleri* (1, 2). In both of these plants, an oleate hydroxylase enzyme catalyzes the hydroxylation chemistry that converts oleate (*cis*-9-octadecenoic acid, or 18:1Δ^9) to ricinoleate (D-12-hydroxyoctadec-*cis*-9-enoic acid, or 12-OH 18:1Δ^9).[1] Subsequent elongation and/or desaturation can give rise to other hydroxy fatty acids such as densipoleate (12-OH 18:2$\Delta^{9,15}$), lesqueroleate (14-OH-20:1Δ^{11}), and auricoleate (14-OH-20:2$\Delta^{11,17}$). The hydroxylase enzymes from castor, CFAH[2] (1), and *Lesquerella*, LFAH (2), are closely related to the common plant oleate desaturase enzyme (FAD2), which converts oleate (18:1 Δ^9) into linoleate (18:2$\Delta^{9,12}$). Indeed, LFAH actually retains both hydroxylase and desaturase activity, indicating that these two oxidation reactions can be catalyzed by the same enzyme. Amino acid sequence alignments (Table I) of these two oleate hydroxylases with several oleate desaturases indicated that there are only a few conserved desaturase residues that are not conserved in the hydroxylases (3). These seven residues (*Arabidopsis thaliana* FAD2 residues 63, 104, 148, 217, 295, 322, and 324)[3] were replaced with the corresponding residues of LFAH, and the resulting enzyme (designated m7FAD2 in Ref. 3) was found to be sufficient to convert the desaturase into a bifunctional desaturase/hydroxylase. A reciprocal experiment in which the desaturase residues were substituted into the LFAH generated an enzyme (designated m7LFAH12 in Ref. 3) with increased desaturase activity, confirming the importance of these residues in specifying the catalytic outcome (3).

The oleate desaturase and oleate hydroxylase enzymes are members of a large class of membrane-bound enzymes that contain a tripartite histidine sequence motif and two putative membrane-spanning domains (4). The enzymes are localized in the endoplasmic reticulum membrane (5) and oxidize oleoylphosphatidylcholine (6) in a reaction that also requires molecular oxygen and reducing equivalents, provided by cytochrome b_5. Members of a family of soluble enzymes that includes fatty acid desaturases (*e.g.* stearoyl-acyl carrier protein Δ^9 desaturase) and hydrocarbon monooxygenases (*e.g.* methane monooxygenase) catalyze a similar array of oxidative chemistry (7,

* This work was supported through the Office of Basic Energy Sciences of the United States Department of Energy and Oilseed Engineering Alliance funded by Dow Chemical and Dow Agrosciences. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed: Dept. of Biology, Bldg. 463, Brookhaven National Laboratory, 50 Bell Ave., Upton, NY 11973. Tel.: 631-344-3414; Fax: 631-344-3407; E-mail: shanklin@bnl.gov.

[1] For fatty acid nomenclature, *X*:*Y* indicates that the fatty acid contains *X* number of carbon atoms and *Y* number of double bonds; Δ^z indicates that a double bond is positioned at the *z*th carbon atom from the carboxyl terminus.

[2] The abbreviations used are: CFAH, *R. communis* oleate Δ^{12} hydroxylase; FAD2, oleate Δ^{12} desaturase; LFAH, *L. fendleri* oleate Δ^{12} hydroxylase/desaturase; GC, gas chromatography; MS, mass spectrometry; L4M, *A. thaliana* FAD2 with four substitutions from *L. fendleri* oleate Δ^{12} hydroxylase/desaturase (A104G/T148N/S322A/M324I); L7M, *A. thaliana* FAD2 with seven substitutions from *L. fendleri* oleate Δ^{12} hydroxylase/desaturase; C4M, *A. thaliana* FAD2 with four substitutions from *R. communis* oleate Δ^{12} hydroxylase (A104G/T148I/S322A/M324V); C2M, *A. thaliana* FAD2 with two substitutions from *R. communis* oleate Δ^{12} hydroxylase; C3M, *A. thaliana* FAD2 with three substitutions from *R. communis* oleate Δ^{12} hydroxylase; C7M, *A. thaliana* FAD2 with seven substitutions from *R. communis* oleate Δ^{12} hydroxylase.

[3] Unless noted, residue numbering is based on the *A. thaliana* FAD2 sequence.

TABLE I

Amino acid comparison of residues that differ between the oleate desaturases and hydroxylases

Residue numbering is based on the *A. thaliana* FAD2 sequence. LFAH and CFAH represent the *L. fendleri* oleate hydroxylase and the castor oleate hydroxylase, respectively. The FAD2 consensus sequence is conserved among the plant FAD2 sequences available in GenBank™. Bold-faced residue numbers are located within five residues of one of the three His clusters that have been proposed to coordinate the nonheme iron active site.

Residue	63	**104**	**148**	217	295	**322**	**324**
FAD2	Ala	Ala	Thr	Tyr	Ala	Ser	Met
LFAH	Val	Gly	Asn	Phe	Val	Ala	Ile
CFAH	Ser	Gly	Ile	Phe	Val	Ala	Val

8). These enzymes utilize a di-iron center to catalyze desaturation or hydroxylation reactions. Despite intense study, there is no definitive evidence to explain the divergent activities of these enzymes that share similar di-iron centers and protein folds. The membrane-bound class of enzymes also contain two catalytically relevant iron atoms per subunit that are proposed to be ligated by the His residues that comprise the characteristic sequence motif (4, 9). The occurrence of highly homologous desaturases and hydroxylases that use the same substrate presents an excellent opportunity to study the molecular determinants of catalytic specificity (desaturation *versus* hydroxylation).

In this study, we report that incorporating a small set of the castor hydroxylase residues into the *Arabidopsis* FAD2 produces an enzyme that performs as well as wild-type hydroxylase enzymes when expressed in *A. thaliana*. Furthermore, the contributions of the individual residues in determining product outcome have been assessed by analyzing a series of single, double, and triple mutants of FAD2 through expression in both *A. thaliana* and *Saccharomyces cerevisiae*.

EXPERIMENTAL PROCEDURES

Site-directed Mutagenesis—Mutations were introduced into *A. thaliana* oleate-12 desaturase (*fad2*) through the use of overlap extension polymerase chain reaction as previously described (3). The oligonucleotides (Invitrogen, Carlsbad, CA) used were D5′, D3′, mD2f, mD2r, mD4f, mD4r, mD5f, mD5r, mD6f, and mD6r (3) as well as mDC1f (gacatcattata**tcc**tcatgcttctact), mDC1r (agtagaagcatga**gga**tataatgatgtc), mDC3f (caccattccaaca**att**ggatccctcgaa), mDC3r (ttcgagggatcca**aat**gttggaatggtg), mDC7f (cacctgttctcgaca**g**t**g**ccgcattataacgc), mDC7r (gcgttataatgcggca**c**tgtcgagaacaggtg), mDC67f (cacctgttc**g**cgaca**g**tgccgcattataacgc), and mDC67r (gcgttataatgcggc**ac**tgtc**gc**gaacaggtg). Mutations were introduced into LFAH with the same overlap extension strategy with the following oligonucleotide pairs: for N149I, LesHF (gatcaagcttatgggtgctggtggaagaataatg) and Les1R (ctcgagagatcc**ta**tgttggaatggtg), and Les1F (caccattccaaca**ta**ggatctctcgag) and LesER (gatcgaattctcataacttattgttgtaatagta); for N149T, LesHF and Les2R (ctcgagagatcc**tg**tgttggaatggtg), and Les2F (caccattccaaca**ca**ggatctctcgag) and LesER. The bold and underlined letters indicate altered nucleotides and codons, respectively.

Expression of Variants in Arabidopsis—fad2 mutants were cloned into the *Sac*I/*Xma*I sites of the *Agrobacterium* binary vector DATNAP, a derivative of pRD410 (10), to direct seed-specific expression from the napin promoter (11). The vectors were introduced into *Agrobacterium tumefaciens* strain GV3101 pMP90 by electroporation and used to transform *A. thaliana* FAD2-deficient plants (12) by the floral dip method (13).

Yeast Expression Conditions—Genes were cloned into pYes-II for expression in *S. cerevisiae* YPH499 (ATCC, Manassas, VA) or INVSCI (Invitrogen) with *Hin*dIII/*Eco*RI sites for the *Lesquerella* mutants or *Acc65I*/*Eco*RI sites for the *fad2* mutants. Upon transformation of the yeast with a lithium acetate method (14), cultures (1 ml) were initially grown on SC−URA medium (yeast synthetic complete medium devoid of uracil, Sigma) supplemented with 1% casamino acids and 2% glucose. Once cells reached an optical density (600 nm) of ~2, the cells were washed with glucose-free medium, resuspended in SC−URA containing 1% casamino acids and 2% galactose, and grown at 30 °C for 48 h. For stereochemistry studies, [12-$^2H_1$](*R*)-stearoyl methyl ester, [12-$^2H_1$](*S*)-stearoyl methyl ester, or [12-$^2H_1$](*S*)-oleoyl methyl ester (the generous gift of Chris Savile and Dr. Peter Buist, Carleton University, Ottawa, Canada) was added as an ethanolic solution to a sterile glass tube. When using the labeled methyl stearate, cerulenin (10 μg/ml, Sigma) and myristic acid (20 μg/ml) were added to the tubes and the ethanolic solvent was removed by evaporation; we found that the presence of ethanol (0.5–1%) substantially decreased hydroxy fatty acid accumulation. SC−URA medium (1 ml) containing 1% casamino acids, 2% glucose (or galactose for induction) and 0.5% Tergitol NP-40 was added to the dry tubes prior to inoculation.

Fatty Acid Analysis—Seeds were methylated (1 ml of 1 N HCl-methanol (Supelco), 80 °C for 1 h), extracted with hexane, and trimethylsilylated (100 μl of BSTFA-TMCS ([*N*,*O*-bis(trimethylsilyl)trifluoroacetamide-trimethylchlorosilane], Supelco), 90 °C for 45 min). The BSTFA-TMCS was removed by evaporation and the sample resuspended in hexane. Yeast pellets were dried by a nitrogen stream prior to methylation, and, when fatty acids were added during growth, cell pellets were washed with 1% Tergitol and then water before drying. Samples were analyzed on a Hewlett-Packard 6890 gas chromatograph equipped with a 5973 mass selective detector (GC/MS) and a J&W DB-23 capillary column (60 m × 250 μm × 0.25 μm). The injector was held at 225 °C, the oven temperature was varied (100–160 °C at 25 °C/min, then 10 °C/min to 240 °C), and a helium flow of 1.1 ml/min was maintained.

RESULTS

Analysis of Variant fad2 Genes—As illustrated in Table I, residues 63, 104, 148, 217, 295, 322, and 324 of FAD2 differ from the corresponding residues found in the closely related LFAH and CFAH. The replacement of all seven (L7M) or a subset of four (104, 148, 322, and 324, L4M) residues of FAD2 with those from LFAH gives rise to variant FAD2 enzymes that catalyze both desaturation and hydroxylation. Because residues 63, 148, and 324 of the *Lesquerella* and castor oleate hydroxylases (*A. thaliana* FAD2 numbering) are different, we also constructed variant *fad2* genes incorporating either seven or four of these substitutions using the corresponding residues of the castor oleate hydroxylase. In an effort to dissect the contribution of individual amino acids in determining product distribution, we constructed a set of variant *fad2* genes containing one, two, or three amino acid substitutions.

The variant *fad2* genes were introduced into *A. thaliana* FAD2-deficient plants by *Agrobacterium*-mediated transformation. Trimethysilylated fatty acid methyl esters from seeds harvested from T2 plants (heterozygous) were analyzed by gas chromatography/mass spectrometry to determine the overall fatty acid composition. The results are shown in Fig. 1. All FAD2 variants expressed in *Arabidopsis* gave detectable amounts of hydroxy fatty acids, ranging from 0.03 to 22%. Furthermore, similar distribution patterns of the hydroxy fatty acids (18:1-OH > 18:2-OH > 20:1-OH ≫ 20:2-OH) were observed in all plants. Interestingly, the wild-type FAD2 enzyme also produced detectable levels of ricinoleic acid (average of 0.03% of total fatty acids). Because ricinoleic acid was detected in seeds of both wild-type and FAD2-deficient plants transformed with *fad2* but not in untransformed *A. thaliana* FAD2-deficient plants, the hydroxylation activity must be associated with FAD2.

Analysis of the transgenic plants expressing FAD2 variants that incorporated substitutions corresponding to their equivalents from the castor hydroxylase gene residues revealed different phenotypes than were observed with the corresponding *Lesquerella* substitutions. The introduction of four amino acid

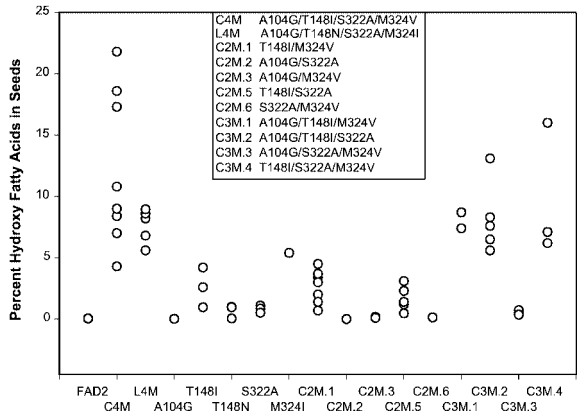

FIG. 1. **Accumulation of hydroxy fatty acids (12-OH 18:1Δ^{9} + 12-OH 18:2$\Delta^{9,15}$ + 14-OH-20:1Δ^{11} + 14-OH-20:2$\Delta^{11,17}$) in seeds of *A. thaliana* FAD2-deficient plants transformed with FAD2 variants under control of the seed-specific napin promoter.** Each *circle* represents data obtained from a single plant.

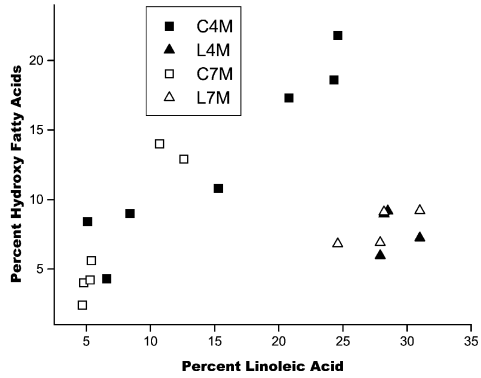

FIG. 2. **Relationship of hydroxy fatty acids and linoleic acid generated by the expression of the FAD2 variants (C4M, C7M, L4M, and L7M) in seeds of *A. thaliana* FAD2-deficient plants.**

substitutions (A104G/T148I/S322A/M324V, or C4M) generated an enzyme that produced levels of hydroxy fatty acids (up to 22%) similar to those obtained upon expression of the wild-type CFAH in *Arabidopsis* FAD2-deficient plants (also under control of the napin promoter) (15). A large range of hydroxy fatty acid product was observed among independent lines, presumably because of differences in the context of the insertion site or the gene copy number. In addition to demonstrating substantial hydroxylase activity, C4M and C7M produced roughly equivalent amounts of linoleic acid and ricinoleic acid in transgenic plants (Fig. 2). When compared with the results obtained upon expression of L4M or L7M, where desaturation dominates hydroxylation, it is clear that the high levels of hydroxy fatty acids observed in several lines of *A. thaliana* FAD2-deficient/C4M plants result from an improved enzyme specificity toward hydroxylation and not simply from optimal transgene context.

Most of the FAD2 variants, with the exception of some of the plants expressing C4M or C7M, retained sufficient desaturase activity to complement the FAD2-deficient background (25–30% linoleic acid). Among the FAD2 variants with single amino acid changes, T148I and M324I caused the most dramatic change in phenotype, producing up to 4.2 and 5.4% hydroxy fatty acids, respectively (Fig. 1). FAD2 variants A104G, S322A, and T148N (a *Lesquerella* substitution) produced less than 1% hydroxy fatty acids. Among the double mutants analyzed, C2M.1 (T148I/M324V) and C2M.5 (T148I/S322A) produced higher levels (4.5 and 3.1%) of hydroxy fatty acids than C2M.2 (A104G/S322A), C2M.3 (A104G/M324V), and C2M.6 (S322A/M324V), which gave less than 0.2%. The triple mutants C3M.1 (A104G/T148I/M324V), C3M.2 (A104G/T148I/S322A), and C3M.4 (T148I/S322A/M324V) generated far more hydroxylated fatty acids (9–16%) than did C3M.3 (A104G/S322A/M324V) (0.7%). Taken together, these data reveal a dominant role of Ile at position 148 in specifying hydroxylation, and indicate that a single amino acid change (M324I) can impart a substantial shift in catalytic specificity.

Given the variability observed between independent transgenic plant lines (Fig. 1), the amount of time required to generate transgenic plants, and our desire to compare the specificity of numerous FAD2 variant enzymes, we have explored the use of *S. cerevisiae* as a complementary host system. The various enzymes were cloned into the pYes-II expression vector behind a GAL-1 promoter as previously described (3). Expression of FAD2 and LFAH was then tested in the yeast strains INVSCI and YPH499 (a strain that Hills and collaborators had reported accumulation of high levels of 18:2 upon expression of a FAD2 enzyme (Ref. 16)) and at multiple temperatures (15, 22, and 30 °C). The highest product accumulation was obtained with the YPH499 strain induced at 30 °C (16). We also found that induction at high starting cell densities led to higher product accumulation; inducing at an A_{600} of 2.5 resulted in the accumulation of 30% diene (from FAD2 expression) or 27% hydroxylated fatty acids (from LFAH expression), as compared with 17 and 18%, respectively, when the cultures were induced at an A_{600} of 0.2.

Expression of the castor oleate hydroxylase under all conditions resulted in cessation of yeast growth, and cultures failed to accumulate detectable product. Because of ricinoleic acid accumulation of up to ~25% upon expression of LFAH, this toxicity cannot be attributed to the accumulation of ricinoleic acid. Table II contains the results of the expression of parental enzymes, quadruple mutants L4M and C4M, all possible triple (C3M) and double (C2M) mutant combinations with the C4M residues, and all single mutants that contain *Lesquerella* or castor substitutions. Although all the enzymes acted upon palmitoleate in addition to oleate, only the data from oleate oxidation is shown because the higher specificity toward oleate permitted more precise measurements of the products obtained from oleate oxidation at lower concentrations. As observed by their transgenic expression in plants, LFAH and FAD2 produce both desaturation and hydroxylation products. A recent publication by Smith *et al.* (17) reported that CFAH also produces linoleic acid when expressed in yeast, albeit to a lesser extent than LFAH. The collection of variant FAD2 enzymes included in Table II contains enzymes exhibiting product ratios intermediate between those of the parental enzymes. There is a strong correlation (r = 0.88) between the hydroxylation specificity observed in yeast and the accumulation of hydroxy fatty acids in plants, suggesting that the information obtained from yeast expression has predictive value regarding relative activity upon expression in *A. thaliana*. Because both ricinoleic acid and linoleic acid are end products in yeast, whereas they are further metabolized in *A. thaliana*, a precise product ratio is obtained more readily from expression in yeast than expression in *A. thaliana*. Although we would expect protein expression levels to have an effect on the amounts of enzymic product observed, the catalytic specificity of the enzymes should not be affected by such variations.

Based on transgenic expression in *S. cerevisiae*, C4M exhibited the highest hydroxylation/desaturation product ratio among all FAD2 variants at 0.55 (the average was 0.91 *in planta*, data from Fig. 2). C4M is a more specific hydroxylase than L4M, and the castor hydroxylase is a more specific hydroxylase than the *Lesquerella* hydroxylase. This implies that at least some of the specificity determinants of the castor hy-

TABLE II

Catalytic specificity of A. thaliana FAD2 and L. fendleri oleate hydroxylase variants as determined by product analysis of S. cerevisiae cultures

The percentage values, indicating the composition in the fatty acid sample, are mean values based on at least three measurements.

Enzyme/variant	FAD2 amino acid substitution(s)	12-OH-18:1Δ^9	18:2$\Delta^{9,12}$	Hydroxylation/ desaturation ± S.E.	Increase[a]
		%	%		*-fold*
FAD2		0.16	26.1	0.006 ± 0.001	1.0
A104G	A104G	0.2	15.5	0.013 ± 0.004	2.1
T148N	T148N	0.22	11.0	0.02 ± 0.002	3.3
T148I	T148I	0.36	4.0	0.09 ± 0.011	14.8
S322A	S322A	0.15	17.8	0.008 ± 0.001	1.3
M324I	M324I	1.6	4.9	0.33 ± 0.07	54.1
M324V	M324V	1.2	3.9	0.30 ± 0.04	49.2
C2M.1	T148I/M324V	0.9	5.6	0.16 ± 0.018	26.2
C2M.2	A104G/S322A	0.063	3.4	0.019 ± 0.002	3.1
C2M.3	A104G/M324V	0.06	3.7	0.016 ± 0.002	2.6
C2M.4	A104G/T148I	1.2	6.6	0.18 ± 0.012	29.5
C2M.5	T148I/S322A	0.76	4.9	0.16 ± 0.001	26.2
C2M.6	S322A/M324V	0.25	19.7	0.013 ± 0.001	2.1
C3M.1	A104G/T148I/M324V	1.8	8.1	0.22 ± 0.024	36.1
C3M.2	A104G/T148I/S322A	1.5	5.5	0.27 ± 0.016	44.3
C3M.3	A104G/S322A/M324V	0.38	14.6	0.026 ± 0.002	4.3
C3M.4	T148I/S322A/M324V	0.82	4.0	0.21 ± 0.021	34.4
L4M	A104G/T148N/S322A/M324I	0.9	6.4	0.15 ± 0.050	24.6
C4M	A104G/T148I/S322A/M324V	1.2	2.2	0.55 ± 0.057	90.2
LFAH		18.2	6.7	2.72 ± 0.2	442.6
LFAH-N149I		12.2	2.4	5.1 ± 1.0	836.1

[a] -Fold increase of hydroxylation over desaturation with respect to FAD2.

droxylase are contained within the C4M residues. Only two of these residues (148 and 324) are different between L4M (Asn-148 and Ile-324) and C4M (Ile-148 and Val-324). Inspection of the product ratios of these four single mutants (Table II) demonstrates that there is little difference between M324V and M324I, whereas there is a 4.5-fold difference between the product ratios of T148N and T148I, suggesting that the T148I mutation is key to determining the product distribution of C4M and the castor hydroxylase.

The results from the single mutants clearly demonstrate that each of the single mutations is sufficient to increase the hydroxylation activity of the FAD2 desaturase. Whereas T148N, A104G, and S322A give modest changes in the product distribution (<3.3-fold increase in hydroxylation specificity), T148I, M324I, and M324V dramatically alter the product distribution (15–54-fold). The T148I, M324I, and M324V mutations affect the accumulation of both desaturated and hydroxylated product. For example, by simply substituting Val for Met at residue 324, the amount of desaturation product decreased 6.7-fold while the amount of hydroxylation product increased ~7.5-fold. Analysis of the double mutants clearly supports the significance of T148I in determining product distribution. All three double mutants that contain T148I exhibit increased hydroxylation and decreased desaturation (26–30-fold increase in hydroxylation specificity over that of FAD2), whereas those that do not contain T148I display reduced desaturation but very little change in hydroxylation (<3.1-fold increase in hydroxylation specificity). Interestingly, the effects of M324V appear to be largely masked when combined with all other mutations; only the T148I/M324V double mutant retains high hydroxylation activity, and this may be attributed to the contribution of T148I. The triple mutants further confirm the importance of T148I, as only those triple mutants that include T148I have markedly increased hydroxylation specificity (25–44-fold increase over FAD2).

Rational Engineering of LFAH—Given the product distribution variability observed when position 148 of FAD2 was changed to the corresponding residue of CFAH or LFAH, we sought to determine whether we could alter the specificity of LFAH by replacing the equivalent residue (Asn-149) with either the FAD2 (Thr) or CFAH (Ile) residue. We found that the reaction specificity of LFAH was modified from a value of 2.7 (hydroxylation:desaturation ratio) to 1.08 when Asn-149 was replaced with Thr (Table III). Replacement of Asn-149 with the CFAH equivalent (Ile) created an enzyme with an increased hydroxylation specificity of 5.1.

Chain Length Affects Catalytic Specificity—As previously reported, the oleate desaturase and hydroxylase enzymes oxidize palmitoleic acid in addition to oleic acid (3, 29). In yeast cells that contain approximately equal quantities of the two monounsaturated fatty acids, FAD2 produces roughly 7.5 times as much 18:2 as 16:2 and LFAH roughly 24 times as much 12-OH 18:1Δ^9 as 12-OH 16:1Δ^9. What is interesting is that the hydroxylation to desaturation ratio for LFAH is quite different for the two substrates, as illustrated in Table III; it is 2.7 for 18:1Δ^9 and 0.29 for 16:1Δ^9, approximately a 10-fold difference. Although this bias could be attributed to differential metabolism of the hydroxy fatty acids, the fact that both of these ratios follow the same trend within the series of *Lesquerella* variants (more hydroxylation for LFAH-N149I, less hydroxylation for LFAH-N149T; data in Table III) suggests that the bias is associated with the mechanism of the partitioning between desaturation and hydroxylation.

Stereospecificity of Variant Enzymes—The catalytic mechanism of the variant FAD2 enzymes was investigated through analysis of the oxidation products of stereospecifically labeled stearate and oleate. Yeast cells expressing FAD2, LFAH, C4M, or FAD2-M324I were grown and induced in the presence of deuterated stearoyl methyl ester ([12-$^2H_1$](*S*)-18:0 or [12-$^2H_1$](*R*)-18:0); the yeast acyl-CoA Δ^9 desaturase enzyme desaturated sufficient quantities of the labeled stearate to provide the necessary labeled oleate for enzymatic desaturation/hydroxylation. Cerulenin was added to the cultures to minimize endogenous fatty acid synthesis so as to prevent dilution of the labeled stearate (18). After 48 h of induction, the cellular fatty acids were analyzed by GC/MS to determine the [^{2}H]/[H] ratio (measured as the ratio of the $M^+ + 1$ peak to the M^+ peak) of the enzymic products (linoleate and ricinoleate). These values were then corrected to account for the contribution of endogenous unlabeled substrate to the peak intensities. From the data shown in Table IV, it is clear that FAD2 and LFAH specifically remove the [12-$^2H_1$](*R*) hydrogen while they

TABLE III

Catalytic specificity of LFAH, LFAH-N149T, and LFAH-N149I as a function of the substrate

Values represent the ratio of hydroxylation product to desaturation product for the given substrate. Standard error is derived from three experiments.

Substrate	Enzyme		
	LFAH	LFAH-N149T	LFAH-N149I
$18{:}1\Delta^9$	2.71 ± 0.20	1.08 ± 0.14	5.1 ± 1.0
$16{:}1\Delta^9$	0.29 ± 0.019	0.21 ± 0.045	0.59 ± 0.12

TABLE IV

Stereospecificity of FAD2, LFAH, FAD2-C4M, and FAD2-M324I as determined by GC/MS analysis of the products formed when using labeled oleate ([12-^{2}H](R)-18:1Δ^9 or [12-^{2}H](S)-18:1Δ^9) as substrate

The values in the table represent the ratio of the M+1 peaks (presence of ^{2}H) to the M^+ peaks (loss of ^{2}H) for the enzymatic products and are corrected for the endogenous unlabeled oleate substrate.

	FAD2 with [12-^{2}H](*R*)18:0[a]	FAD2 with [12-^{2}H](*S*)18:0[a]	LFAH with [12-^{2}H](*R*)18:0[a]	LFAH with [12-^{2}H](*S*)18:0[a]	LFAH with [12-^{2}H](*S*)18:1Δ^9	C4M with [12-^{2}H](*S*)18:1Δ^9	M324I with [12-^{2}H](*S*)18:1Δ^9
$18{:}2\Delta^{9,12}$	0.08	0.77	0.06	0.84	0.77	0.66	0.67
12-OH $18{:}1\Delta^9$	ND[b]	ND	0.03	1.08	0.63	0.73	0.54

[a] Exogenous 18:0 was converted to $18{:}1\Delta^9$ through the action of endogenous acyl-CoA desaturase.
[b] ND, not determined.

retain the [12-^{2}H$_1$](*S*) hydrogen; this result is consistent with the known stereochemistry of FAD2 (19), LFAH (20), and CFAH (21). Although these growth conditions permitted incorporation of high levels of the labeled stearoyl methyl ester, product accumulation was decreased markedly, preventing analysis of the less active FAD2 variants.

To characterize the specificity of the FAD2 variants C4M and M324I, yeast cells were grown and induced in the presence of labeled oleate ([12-^{2}H$_1$](*S*)-18:1Δ^9). The addition of this unsaturated fatty acid partially attenuates endogenous unsaturated fatty acid synthesis, and thus cerulenin was not required (22). Again, GC/MS was employed to determine the [^{2}H]/[H] ratio of the enzymic products (linoleate and ricinoleate). Furthermore, the [^{2}H]/[H] data for the ricinoleic acid products have been confirmed using matrix-assisted laser desorption ionization time-of-flight mass spectrometry (23). The values presented in Table IV demonstrate that LFAH, FAD2-C4M, and FAD2-M324I retain the 12(*S*)-hydrogen upon formation of both $18{:}2\Delta^{9,12}$ and 12-OH $18{:}1\Delta^9$. At this time we cannot formally rule out the possibility that an intramolecular isotope effect could have biased the reaction in favor of the observed results. However, this seems unlikely because the oxidation of [12-^{2}H$_1$](*R*)-18:1Δ^9 and [12-^{2}H$_1$](*S*)-18:1Δ^9 by LFAH gave the expected products. Nonetheless, the available data indicate that the stereospecificity of the FAD2 variants is consistent with that of the characterized FAD2 (19), LFAH (20), and CFAH (21).

Generality of Bifunctional Activity—The surprising finding that the wild-type *A. thaliana oleate* desaturase had detectable hydroxylase activity prompted us to investigate the generality of bifunctional activity among other membrane-bound fatty acid desaturases. We first examined the seed oil of a number of plants to determine whether ricinoleic acid accumulated as a result of a bifunctional FAD2 enzyme. *Arabidopsis* oil, olive oil, flax oil, and soybean oil all contained measurable amounts (~0.015%) of ricinoleic acid, indicating that the FAD2 enzymes found in these plants may also be bifunctional.

In addition to ricinoleic acid, detectable levels of a novel hydroxylated fatty acid were detected in flax seed oil (Fig. 3, *peak g*). Using GC/MS detection, this analyte displayed a major ion at 145 *m/z* which is consistent with a $(CH_3)_3SiOCH(CH_2)_2CH_3$ ion. Smaller fragments at 73 *m/z* ($OSi(CH_3)_3$) and 310 *m/z* (M-($OSi(CH_3)_3$)) are also present. These fragments are consistent with a description of the analyte as 15-hydroxylinoleate; the major fragment would arise from cleavage adjacent to the carbon bearing the oxygen, between C-14 and C-15 (15). This analyte eluted from the GC column at a time consistent with such an assignment. The retention time of ricinoleate (12-hydroxyoleate) is ~0.3 min greater than that of linoleate, whereas the novel analyte eluted ~0.3 min after linolenate (Fig. 3, *peak f*). We propose that this fatty acid may arise through the action of a bifunctional linoleate desaturase (*e.g.* FAD3), although these data do not conclusively rule out alternative explanations for the origin of this fatty acid. It is likely that we were able to observe the 15-hydroxylinoleate in flax seed because flax accumulates high levels of linolenate. The activity of this enzyme or at least the flux through the linoleate desaturase is sufficient to allow detectable quantities of this unusual fatty acid to accumulate.

We next tested the *S. cerevisiae* Δ^9 acyl-CoA desaturase, an enzyme that shares a histidine sequence motif and predicted membrane topology, but shares only ~25% sequence identity with the *A. thaliana* FAD2 (4). GC/MS analysis of the trimethylsilyl derivatives of fatty acid methyl esters from wild-type yeast (strains INVSCI and YPH499) revealed the presence of small quantities (0.2–1% of total fatty acids) of 9-hydroxypalmitate (MS fragment ions at 201 and 259 *m/z*) and 9-hydroxystearate (MS fragments at 229 and 259 *m/z*). Analysis of the fatty acids from an L814C (a desaturase null strain that is an unsaturated fatty acid auxotroph (Ref. 24)) culture grown in the presence of palmitoleate and oleate revealed no detectable hydroxy fatty acids. Transformation of this strain with a vector containing the gene for the stearoyl-CoA Δ^9 desaturase from rat (25) or fruit fly (*Drosophila melanogaster* CS strain, des 1 gene) (26) complemented the unsaturated fatty acid auxotrophy of the strain. In addition to producing the expected palmitoleate and oleate, these enzymes produced detectable levels of 9-hydroxypalmitate and 9-hydroxystearate similar to those found in wild-type yeast.

Finally, the *Bacillus subtilis* Δ^5 desaturase (27) was expressed in *Escherichia coli* BL21(DE3) and found to produce trace quantities of 5-hydroxy palmitate (MS fragment ions of 203 and 257 *m/z*) in addition to its primary desaturation product,$16{:}1\Delta^5$. These fragment ions were not detected from *E. coli* BL21(DE3) cells containing a pET-3a vector lacking a desaturase. We did not identify any hydroxy fatty acids in the linoleic acid-producing yeasts (28) *Rhodotorula glutinis* or *Cryptococcus laurentii*, indicating either that the oleate desaturase from these organisms does not have any hydroxylase activity or that these particular yeast have an efficient mechanism for metabolizing hydroxylated fatty acids.

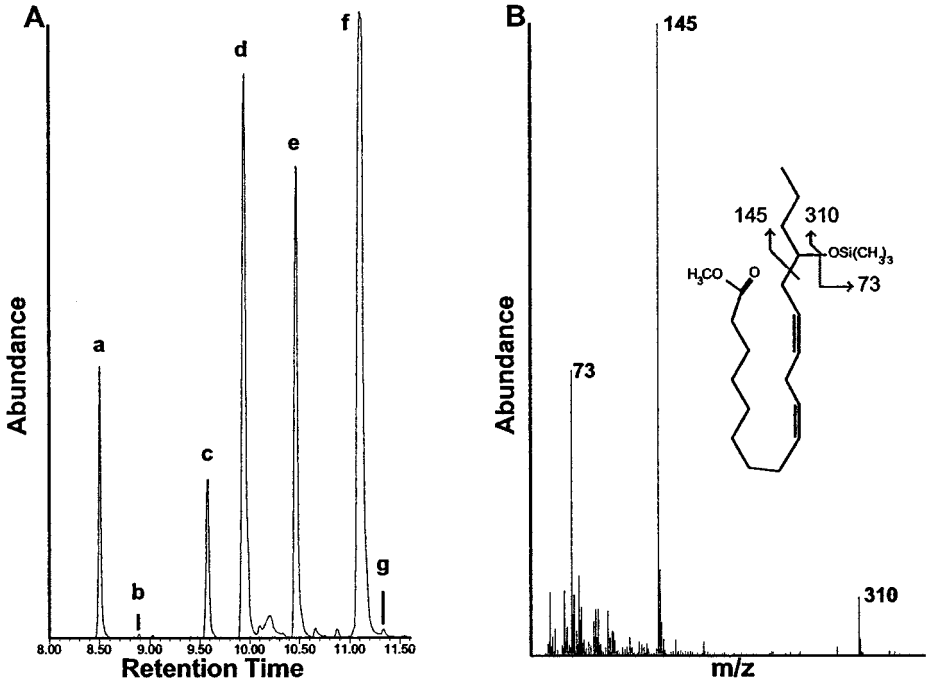

FIG. 3. *A*, GC/MS chromatogram of the trimethylsilyl ether derivatives of fatty acid methyl esters of flax seed oil: *peak a*, 16:0; *peak b*, 16:1Δ^9; *peak c*, 18:0; *peak d*, 18:1Δ^9; *peak e*, 18:2$\Delta^{9,12}$; *peak f*, 18:3$\Delta^{9,12,15}$; *peak g*, 15-OH-18:2$\Delta^{9,12}$. *B*, mass spectrum of *peak g*, with diagnostic fragmentation ions identified.

DISCUSSION

The plant oleate desaturase and oleate hydroxylase enzymes are closely related enzymes exhibiting diverged function. We have reported that the sequence determinants for fatty acid hydroxylation are largely confined to a small set of residues that are located adjacent to the proposed iron ligands (3). Through the analysis of the products obtained by expression of variant FAD2 enzymes in both *A. thaliana* and *S. cerevisiae*, we have dissected the role of each of these four residues in determining enzyme function. Analysis of the data obtained from *Arabidopsis* expression indicates several important points. First, C4M is capable of generating levels of hydroxy fatty acids (22%) in transgenic *A. thaliana* previously observed only with expression of wild-type CFAH (15) and LFAH (2). Second, the identity of the residue at position 148 of FAD2 (equivalent to LFAH-149 or CFAH-152) is important for specifying enzyme function. Third, substantial hydroxy fatty acid accumulation (~5%) can be achieved by the introduction of at least two different single mutations (T148I and M324I) into FAD2. Variability among independent transgenic plants expressing the same construct made it difficult to evaluate the effects of different constructs. We therefore explored the possibility of analyzing the constructs in yeast to overcome this problem.

The utility of *S. cerevisiae* as a host for the functional heterologous expression of plant fatty acid desaturases is well established (3, 16, 29). For example, expression of FAD2 desaturases resulted in linoleic acid accumulation of up to ~40%. However, expression of CFAH and LFAH has been less successful, with accumulation of no more than 2% hydroxy fatty acids (3, 17). We determined that by using an appropriate host strain (*S. cerevisiae* YPH499) and expression conditions (30 °C induction at high cell density) we were able to optimize hydroxy fatty acid accumulation (up to 27% ricinoleic acid) upon expression of LFAH. The ability to accumulate high levels of hydroxy fatty acids in yeast permitted our comparative analysis of the FAD2 variants and could provide a convenient system in which to screen for additional fatty acid hydroxylases.

Yeast, unlike *Arabidopsis*, accumulates linoleic and ricinoleic acids without further metabolism, thereby simplifying analysis because product ratios are a meaningful measurement of enzyme function. Although we have observed minor alterations in product distribution under different growth conditions, the product ratios of ricinoleic acid:linoleic acid are reproducible when identical growth conditions are employed and were found to vary from ~0.0061 for the parental FAD2 enzyme to 2.7 for the wild-type LFAH. The single, double, triple, and quadruple mutants of FAD2 were found to have product ratios nearly spanning this entire range, from 0.008 (S322A) to 0.55 (C4M). Consistent with the *Arabidopsis* expression data, two important observations emerge. First, the substitution of Thr-148 with the castor residue Ile has a strong influence on the enzyme function and is observed in all mutants; those FAD2 variants containing this mutation have product ratios of 0.09–0.55, whereas those that do not have ratios of 0.008–0.14. Second, the single mutants M324V and M324I have product ratios nearly identical to that of the quadruple mutant C4M. Adding additional mutations lessens this effect; however, the positive effects produced by a single residue alteration are commonly masked by additional amino acid substitutions (30). Nonetheless, the ability to alter this product distribution ~49-fold with a single amino acid (effected by a single base change) is substantial. The relative specificities of the *Arabidopsis*, *Lesquerella*, and castor enzymes can be mimicked by the choice of residue at position 148 of FAD2. Substitution of the desaturase residue (Thr) to that of *Lesquerella* (Asn) improves the hydroxylation specificity modestly, whereas substitution with the castor residue (Ile) further increases specificity. Because the effect of the T148I substitution was found to be additive in combination with A104G, S322A, and/or M324V, we predicted that we could decrease the hydroxylation specificity of the *Lesquerella* hydroxylase by making N149T (desaturase change) or increase the specificity by making N149I (castor change). These predictions were borne out, and we found that a single (desaturase) change reduced the hydroxylation specificity ~3-fold, whereas the castor change increased the specificity ~2-fold. Our ability to rationally engineer the specificity of the wild-type LFAH demonstrates the utility of our approach to identify the mutations responsible for determining the enzymatic function.

Both LFAH (2) and CFAH (17) enzymes catalyze desaturation in addition to hydroxylation, suggesting that these enzymes utilize specialized variations of the common desaturase

mechanism. In this report we show that FAD2 also catalyzes oleate hydroxylation, further supporting the relationship between the oleate desaturases and hydroxylases. The presence of trace amounts of ricinoleic acid in the seed oils of soybean (family Fabaceae), olive (family Oleaceae), and flax (family Linaceae) points to a general bifunctional nature of the plant FAD2 enzymes.

The ability to convert the oleate desaturase into a bifunctional desaturase/hydroxylase with as few as one base substitution validates the notion that evolution of the oleate hydroxylase could have progressed incrementally via gene duplication and mutagenesis. The ease of this conversion is reflected by the independent evolution of 12-hydroxylase activity at least several times (1, 2). We have shown that highly divergent desaturases with different regiospecificities retain a similar ability to form small quantities of hydroxylated fatty acids. Despite their limited sequence homology, these classes of membrane-bound di-iron desaturases share the canonical histidine boxes that likely act as the iron ligands as well as predicted transmembrane segments (4). Because we have identified residues adjacent to these conserved histidine clusters that are critical determinants of hydroxylation function, it is conceivable that desaturases with different regiospecificities (31) could become hydroxylases through a similar evolutionary process. The intrinsic hydroxylation activity of desaturases with different regiospecificities makes these enzymes possible targets for future directed evolution experiments. Furthermore, the observation of low level hydroxylation activity at C-12 for the Δ^{12} desaturase from *A. thaliana*, C-9 for the Δ^{9} desaturase of *S. cerevisiae*, C-5 for the Δ^{5} desaturase of *B. subtilis*, and C-15 for the ω-3 linoleate desaturase from flax suggests that these positions are the sites of initial oxidation of the desaturation reactions, corroborating the kinetic isotope effect studies of Buist and coworkers (19, 32–34).

There are many examples of seed oils that contain hydroxy fatty acids other than ricinoleic acid or its derivatives (35). Conacher and Gunstone (36) suggested that the conjugated hydroxy acids such as helenynolic (9-OH,10t,12a-18:2) and dimorphecolic acids (9-OH,10t,12t-18:2) may be biosynthesized by base-catalyzed rearrangement of conjugated epoxide fatty acids. However, the nonconjugated hydroxy acids (*e.g.* isoricinoleic (9-OH 18:1Δ^{12}) or jalapinolic (11-OH 16:0)) may be synthesized by alkene hydration or by hydroxylation catalyzed by cytochrome P450 or diiron enzymes. Given the inherent hydroxylation function of a diverse group of fatty acid desaturases from mammals, insects, fungi, bacteria, and plants, the evolution of fatty acid hydroxylases of varying regiospecificities from these ancestral diiron desaturases certainly seems plausible.

Studies by Morris (21) and Buist and co-workers (19, 37) have shown that the Δ^{12} desaturases and hydroxylases are mechanistically similar; both enzymes specifically remove the pro-*R* hydrogen from C-12 of oleate en route to product formation. The *Lesquerella* enzyme must also share this same stereospecificity, as the seed oil-derived lesquerolic acid retains the same optical rotation properties as ricinoleic acid derived from castor seed oil (20). The knowledge that these enzymes are highly homologous (1, 2) and that the enzymes catalyze both desaturation and hydroxylation, just with differing product ratios, implies that these enzymes employ closely related catalytic mechanisms. We sought to determine the stereospecificity of our variant FAD2 enzymes to gain insight into understanding the cause of bifunctional behavior. Analysis of enzymatically derived products obtained from yeast cultures expressing active FAD2, LFAH, C4M, and FAD2-M324I revealed comparable retention of the 12(*S*)-hydrogen atom. Thus, the variant FAD2 oleate hydroxylases C4M and M324I retain

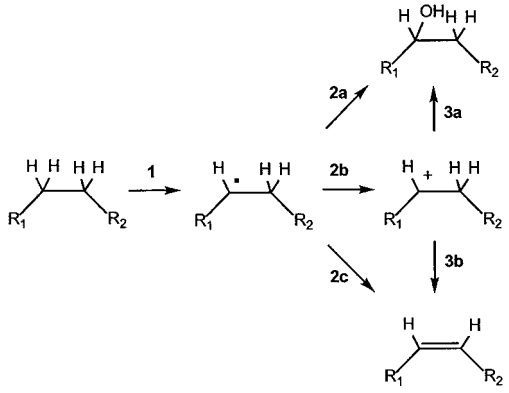

FIG. 4. **Proposed mechanism of hydroxylation and desaturation of fatty acids, adapted from Buist (19).** Upon H-atom abstraction (*1*), desaturation can be achieved through a second H-atom abstraction step (*2c*) or by electron transfer and deprotonation (*2b*, *3b*). Hydroxylation could occur by capture of either the radical (*2a*) or cationic (*3a*) intermediates.

the stereospecificity of the wild-type desaturase and hydroxylase enzymes. The retention of stereospecificity throughout the oleate desaturases and hydroxylases, including both wild-type and variant enzymes, is consistent with tight control of substrate-binding conformation. However, the ability to form distinct products from one enzyme indicates that some flexibility of substrate binding modes, whether static or dynamic, may still exist. The subtlety of the changes necessary to alter the reaction outcome presented here (*e.g.* Met to Ile at position 324), as well as the large number of different changes that can affect reaction outcome, is consistent with our hypothesis that minor alterations in the geometry of the active site can explain the change in function (3).

Recent studies have shown that the chemical nature of the substrate can also influence reaction partitioning of binuclear iron hydroxylases. A mechanistic study of methane monooxygenase hydroxylase demonstrated that the same enzyme oxidant was capable of hydroxylating and desaturating an alternative substrate ethyl benzene (38). The extent of desaturation was found to be dependent on reaction conditions, likely resulting from alteration of substrate positioning. In this experiment, it is believed that resonance stabilization of a radical or cation intermediate, afforded by an aromatic ring, would increase the intermediate lifetime and thereby increase the likelihood of desaturation via reaction pathway partitioning (*steps 2c* and *3b* of Fig. 4). Similarly, a binuclear iron model compound catalyzes desaturation in addition to hydroxylation when presented with a substrate that has the potential to stabilize a radical or cation intermediate (39). In a theoretical study of methane monooxygenase hydroxylase, the activation energy for hydroxylation was found to be minimal (<3.9 kcal/mol) so long as certain geometrical constraints were maintained (40). Taken together, these studies imply that the default activity of an activated binuclear iron center toward an unactivated hydrocarbon substrate is hydroxylation. The catalytic function of a binuclear iron center might be changed to desaturation through alteration of the chemical nature of the substrate (effected by intermediate stabilization) or by substrate presentation to the oxidant.

Although hydrocarbon hydroxylases are capable of controlling the substrate orientation to some degree, small substrate size may also favor hydroxylation (41). The unactivated nature of the desaturase substrates suggests that these enzymes do not use intramolecular intermediate stabilization as a means of achieving desaturation. Perhaps the large size of the fatty acid substrate of the soluble and membrane-bound desaturases

would permit these enzymes the control, as mediated through extensive protein-substrate interactions, necessary to avoid hydroxylation and instead catalyze desaturation. In fact, the ability of the LFAH enzyme to link substrate identity ($16{:}1\Delta^9$ *versus* $18{:}1\Delta^9$) to functional outcome further supports the notion that presentation of the substrate to the oxidant is a critical factor in specifying hydroxylation or desaturation. Development of a crystallographic model of a member of this family of enzymes, and perhaps applying the tools of density functional theory, could greatly assist in our interpretation of these data.

Acknowledgments—We thank Dr. P. Buist and C. Savile for providing the deuterated substrates, Dr. C. Martin for the *S. cerevisiae* L814C strain, Dr. C. Wicker-Thomas for the *D. melanogaster* desaturase gene, Dr. J. Cronan for the *B. subtilis* desaturase gene, and Dr. J. Setlow for editorial assistance.

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Desaturation and Hydroxylation: RESIDUES 148 AND 324 OF ARABIDOPSIS FAD2, IN ADDITION TO SUBSTRATE CHAIN LENGTH, EXERT A MAJOR INFLUENCE IN PARTITIONING OF CATALYTIC SPECIFICITY

John A. Broadwater, Edward Whittle and John Shanklin

J. Biol. Chem. 2002, 277:15613-15620.
doi: 10.1074/jbc.M200231200 originally published online February 25, 2002
