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*

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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 to create 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 b5. Members of a family of soluble enzymes that includes fatty acid desaturases (e.g. stearoyl-acyl carrier protein Δ9 desaturase) and hydrocarbon monoxygenases (e.g. methane monooxygenase) catalyze a similar array of oxidative chemistry (7, 8).

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 18:1Δ9) to ricinoleate (p-12-hydroxyoctadec-9-enoic acid, or 12-0H18:1Δ9).1 Subsequent elongation and/or desaturation can give rise to other hydroxy fatty acids such as densipoleate (12-0H18:2Δ9,12), lesqueroleate (14-0H20:1Δ11), and auricoleate (14-0H20:2Δ11,17). The hydroxylase enzymes from castor, CFAH2 (1), and Lesquerella, LFAH (2), are closely related to the common plant oleate desaturase enzyme (FAD2), which converts oleate (18:1 Δ9) into linoleate (18:2Δ9,12). 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 b5. Members of a family of soluble enzymes that includes fatty acid desaturases (e.g. stearoyl-acyl carrier protein Δ9 desaturase) and hydrocarbon monoxygenases (e.g. methane monooxygenase) catalyze a similar array of oxidative chemistry (7, 8).

Footnotes:
1 For fatty acid nomenclature, X,Y indicates that the fatty acid contains X number of carbon atoms and Y number of double bonds; Δz indicates that a double bond is positioned at the zth carbon atom from the carboxyl terminus.
2 The abbreviations used are: CFAH, R. communis oleate Δ12 hydroxylase/FAO2, oleate Δ12 desaturase; LFAH, L. fendleri oleate Δ12 hydroxylase/desaturase; GC, gas chromatography; MS, mass spectrometry; L4M, A. thaliana FAD2 with four substitutions from L. fendleri oleate Δ12 hydroxylase/desaturase; A104G/T148I/S322A/M324I; L7M, A. thaliana FAD2 with seven substitutions from L. fendleri oleate Δ12 hydroxylase/desaturase; C4M, A. thaliana FAD2 with four substitutions from R. communis oleate Δ12 hydroxylase (A104G/T148I/S322A/M324I); C2M, A. thaliana FAD2 with two substitutions from R. communis oleate Δ12 hydroxylase; C3M, A. thaliana FAD2 with one substitution from R. communis oleate Δ12 hydroxylase; C6M, A. thaliana FAD2 with four substitutions from R. communis oleate Δ12 hydroxylase; C7M, A. thaliana FAD2 with seven substitutions from R. communis oleate Δ12 hydroxylase.
3 Unless noted, residue numbering is based on the A. thaliana FAD2 sequence.
Catalytic Specificity Determinants in Desaturases/Hydroxylases

TABLE I

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

<table>
<thead>
<tr>
<th>Residue</th>
<th>FAD2</th>
<th>LFAH</th>
<th>CFAH</th>
</tr>
</thead>
<tbody>
<tr>
<td>63</td>
<td>Ala</td>
<td>Ala</td>
<td>Thr</td>
</tr>
<tr>
<td>104</td>
<td>Val</td>
<td>Gly</td>
<td>Asn</td>
</tr>
<tr>
<td>148</td>
<td>Ser</td>
<td>Gly</td>
<td>Ile</td>
</tr>
<tr>
<td>217</td>
<td>Tyr</td>
<td>Ala</td>
<td>Ser</td>
</tr>
<tr>
<td>295</td>
<td>Phe</td>
<td>Val</td>
<td>Ala</td>
</tr>
<tr>
<td>322</td>
<td>Phe</td>
<td>Val</td>
<td>Ala</td>
</tr>
<tr>
<td>324</td>
<td>Phe</td>
<td>Val</td>
<td>Ala</td>
</tr>
</tbody>
</table>

Residue numbering is based on the A. thaliana FAD2 sequence. LFAH and CFAH represent the L. fendleri olate hydroxylase and the castor olate 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.
substitutions (A104G/T148I/S322A/M324V, or C4M) generated an enzyme that produced levels of hydrox 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 hydrox 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 hydrox 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 M324V caused the most dramatic change in phenotype, producing up to 4.2 and 5.4% hydrox 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, C2M1 (T148I/M324V) and C2M5 (T148I/S322A) produced higher levels (4.5 and 5.1%) of hydrox fatty acids than C2M2 (A104G/S322A), C2M3 (A104G/M324V), and C2M6 (S322A/M324V), which gave less than 0.2%. The triple mutants C3M1 (A104G/T148I/M324V), C3M2 (A104G/T148I/S322A), and C3M4 (T148I/S322A/M324V) generated far more hydroxylated fatty acids (9–16%) than did C5M3 (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 (M324V) 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 (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 A600 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 A600 of 0.2.

Expression of the castor olate 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 olate, only the data from olate oxidation is shown because the higher specificity toward olate permitted more precise measurements of the products obtained from olate 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-
hydroxylation and decreased desaturation (26–30-fold increase in hydroxylation activity of the FAD2 desaturase). Whereas those that do not contain T148I display reduced desaturation but very little change in hydroxylation (<3.3-fold increase in hydroxylation specificity over that of FAD2), whereas those that contain approximately equal quantities of the two mono-unsaturated fatty acids, FAD2 produces roughly 7.5 times as much 18:2 as 16:2 and LFAH roughly 24 times as much 12-OH 18:1 as 16:2. 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 18:1 and 0.29 for 16:1, 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 ([12-2H1](-18:0); the yeast acyl-CoA dehydratase [12H]/[H] ratio (measured as the ratio of the M⁺ to the M⁺ peak) of the enzyme 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 [12-2H1]/(R) hydrogen while they
The values in the table represent the ratio of the M+1 peaks (presence of 2H) to the M− peaks (loss of 2H) for the enzymatic products and are corrected for the endogenous unlabeled oleate substrate.

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 ([12-2H1](R)-18:1Δ9 or [12-2H1](S)-18:1Δ9) as substrate

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Enzyme</th>
<th>(\text{FAD2 with [12-2H1]R(18:0)})</th>
<th>(\text{FAD2 with [12-2H1]S(18:0)})</th>
<th>(\text{LFAH with [12-2H1]R(18:0)})</th>
<th>(\text{LFAH with [12-2H1]S(18:0)})</th>
<th>(\text{FAD2-M324I with [12-2H1]S(18:1Δ3)})</th>
<th>(\text{C4M with [12-2H1]S(18:1Δ3)})</th>
</tr>
</thead>
<tbody>
<tr>
<td>18:1Δ9</td>
<td>LFAH</td>
<td>2.71 ± 0.20</td>
<td>1.08 ± 0.14</td>
<td>5.1 ± 1.0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>16:1Δ9</td>
<td>LFAH</td>
<td>0.29 ± 0.019</td>
<td>0.21 ± 0.045</td>
<td>0.59 ± 0.12</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
| **Note**: Exogenous 18:0 was converted to 18:1Δ9 through the action of endogenous acyl-CoA desaturase.

To characterize the specificity of the FAD2 variants C4M and M324I, yeast cells were grown and induced in the presence of labeled oleate ([12-2H1](S)-18:1Δ9). 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 \([3\text{H}]/[\text{H}]\) ratio of the enzymic products (linoleate and ricinoleate). Furthermore, the \([\text{H}]/[\text{H}]\) 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 \([12-\text{H}2](\text{S})\) 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.

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**Generality of Binormal Functionality**—The surprising finding that the wild-type A. thaliana oleate desaturase had detectable hydroxylase activity prompted us to investigate the generality of binormal functionality 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 binormal 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 binormal.

In addition to ricinoleic acid, detectable levels of a novel hydroxylated fatty acid were detected in flax seed oil (Fig. 3, peak f). Using GC/MS detection, this analyte displayed a major ion at 145 m/z which is consistent with a \((\text{CH}_3)_3\text{SiOCH(\text{CH}_2)}_2\text{CH}_3\) ion. Smaller fragments at 73 m/z \((\text{OSi(\text{CH}_3)}_3)\) and 310 m/z \((\text{M-OSi(\text{CH}_3)}_3)\) 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-hydroxymyelate) 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 flav 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 Δ9 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-hydroxymyelate (MS fragment ions at 201 and 259 m/z) and 9-hydroxyoleate (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 Δ9 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-hydroxymyelate and 9-hydroxyoleate similar to those found in wild-type yeast.

Finally, the Bacillus subtilis Δ5 desaturase (27) was expressed in Escherichia coli BL21(DE3) and found to produce trace quantities of 5-hydroxymyelate (MS fragment ions of 203 and 257 m/z) in addition to its primary desaturation product, 16:1Δ5. 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.
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 function.

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, 16:1Δ9; peak c, 18:0; peak d, 18:1Δ9; peak e, 18:2Δ9,12; peak f, 18:3Δ9,12,15; peak g, 15-OH-18:2Δ9,12. B, mass spectrum of peak g, with diagnostic fragmentation ions identified.
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 co-workers (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 dimorphicolic 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. isorici-noleic (9-OH:18:1Δ^{2}) 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 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 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 monoxygenase 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 monoxygenase 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

![Fig. 4. Proposed mechanism of hydroxylation and desaturation of fatty acids, adapted from Buist (19).](image-url)
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.

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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

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