

Engineering Δ^9 -16:0-Acyl Carrier Protein (ACP) Desaturase Specificity Based on Combinatorial Saturation Mutagenesis and Logical Redesign of the Castor Δ^9 -18:0-ACP Desaturase*

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Six amino acid locations in the soluble castor Δ^9 -18:0-acyl carrier protein (ACP) desaturase were identified that can affect substrate specificity. Combinatorial saturation mutagenesis of these six amino acids, in conjunction with selection, using an unsaturated fatty acid auxotroph system, led to the isolation of variants with up to 15-fold increased specific activity toward 16-carbon substrates. The most improved mutant, com2, contained two substitutions (T117R/G188L) common to five of the 19 complementing variants subjected to further analysis. These changes, when engineered into otherwise wild-type 18:0-ACP desaturase to make mutant 5.2, produced a 35-fold increase in specific activity with respect to 16-carbon substrates. Kinetic analysis revealed changes in both k_{cat} and K_m that result in an 82-fold improvement in specificity factor for 16-carbon substrate compared with wild-type enzyme. Improved substrate orientation apparently compensated for loss of binding energy that results from the loss of desolvation energy for 16-carbon substrates. Mutant 5.2 had specific activity for 16-carbon substrates 2 orders of magnitude higher than those of known natural 16-carbon specific desaturases. These data support the hypothesis that it should be possible to reengineer archetypal enzymes to achieve substrate specificities characteristic of recently evolved enzymes while retaining the desired stability and/or turnover characteristics of a parental paralog.

Plant-soluble fatty acid desaturase enzymes introduce a double bond regioselectively into a saturated acyl-ACP substrate. The reaction involves activation of molecular oxygen by a two-electron reduced diiron center coordinated by a four-helix bundle that forms the core of the desaturase architecture (1, 2). The archetype of this class is the Δ^9 -18:0-ACP desaturase required by all plants for the maintenance of membrane fluidity (2, 3). While this enzyme primarily desaturates stearoyl-ACP, it is also active to a minor extent with palmitoyl-ACP (4, 5). The crystal structure of the native desaturase revealed a pocket capable of accommodating an 18-carbon substrate in the extended conformation adjacent to the diiron active site (6). While

a pocket capable of binding 18-carbon substrates could also structurally accommodate 16- and 14-carbon substrates, reduced turnover with 16- and 14-carbon substrates has been correlated with a loss of binding energy corresponding to the desolvation of two or four fewer methylene groups (7).

Several naturally occurring variant desaturase enzymes have been isolated from tissues that accumulate unusual fatty acids based on their homology to the archetype 18:0-ACP² desaturases (5, 8–11). The specific activity profiles of these enzymes are consistent with a role of producing the corresponding unusual fatty acids. However, they exhibit very poor specific activities compared with all stearoyl-ACP desaturases reported to date and have proved ineffective in producing altered fatty acid phenotypes when heterologously expressed (Ref. 12 and data not shown). Recent observations from the Ohlrogge group suggests that vegetative ferredoxins,³ and specific ACP isoforms may be required for optimal activity of the variant desaturases (13, 14). In addition to showing poor activities, variant desaturases tended to form insoluble aggregates when purified. Low stability and poor catalytic rates are properties shared by many newly evolved enzymes that arise as gene duplication events in which selection for stability and/or turnover is relaxed, while mutations accumulate that finally result in an alteration of function (15, 16). A prediction of this model is that it should be possible to redesign archetypal enzymes that exhibit desired stability and/or turnover to achieve an altered substrate specificity while retaining the desired properties of the parental paralog.

Bacterial unsaturated fatty acid auxotrophs have been used for the isolation and characterization of both membrane-bound and soluble fatty acid desaturase systems (17–19). We recently described a bacterial selection system capable of distinguishing between soluble desaturase enzymes with different chain length specificities (19). Soluble desaturases specific for 18-carbon chain length are unable to complement MH13, an unsaturated fatty acid auxotroph of *Escherichia coli* defective in the anaerobic dehydratase/isomerase pathway of double bond incorporation into fatty acids, presumably due to the lack of available 18:0-ACP substrate. However, desaturase variants capable of recognizing substrates of 16- or 14-carbon chain length are able to complement the unsaturated fatty acid auxotrophy. To test the selection system, we identified four amino acid positions at the base of the substrate-binding cavity (114,⁴ 118, 179, and 188) and constructed libraries of mutants saturated for substitutions at each of the locations individually.

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¹ Fatty acid nomenclature: X:Y indicates that the fatty acid contains X numbers of carbon atoms and Y numbers of double bonds; Δ^z indicates that a double bond is positioned at the zth carbon atom from the carboxyl terminus.

² The abbreviations used: ACP, acyl carrier protein; wt, wild-type; PCR, polymerase chain reaction.

³ All specific activities compared in this report were determined using *Anabaena* vegetative ferredoxin.

⁴ Amino acid numbering corresponds to the sequence of the mature castor Δ^9 -18:0-ACP desaturase (6).

TABLE I
Specific activity of various single-site mutants of the castor Δ^9 -18:0-ACP desaturase relative to wt

Position	Substitution by random point mutagenesis	Substrate chain length		Substitution by saturation mutagenesis	Substrate chain length	
		14	16		14	16
M114	Ile	4 ^a	2	Phe	6	0.1
T117	Leu	1	0.5	Arg	4	3
L118	Phe	1	4	Phe	1	4
P179	Leu	19	2	Ile	23	7
T181	Leu	1	4	Trp	31	2
G188	— ^b	—	—	Leu	14	15

^a Specific activities for wt are 0.8 and 11.5 nm/min/mg for 14:0- and 16:0-ACP, respectively, at 1 μ M initial substrate concentration.

^b No mutations were identified.

Variants best able to complement the unsaturated fatty acid auxotrophy were isolated and characterized. The resulting enzymes, like those derived from rational design efforts, showed relatively poor specific activity compared with the wt enzyme with its preferred substrate, 18:0-ACP (19, 20). This result is consistent with the observation that hydrophobic partitioning plays a substantial role in the selectivity and reaction rate for the wt castor Δ^9 -18:0-ACP desaturase (7). This conclusion, along with the results of our previous efforts in producing enzymes with desired specificity but poor turnover rates, suggested that this physico-chemical parameter might place an upper boundary to the reaction rate of desaturase with the desired 16-carbon substrate and that it might not be possible to engineer a 16:0- Δ^9 -desaturase with catalytic properties similar to those of the wt enzyme with its preferred substrate.

However, small changes in relative positioning of substrate relative to the active site have been shown to exert large effects on catalytic rates (21, 22). We therefore used combinatorial saturation mutagenesis at positions known to affect substrate specificity to maximize the likelihood of identifying variants with desired improvement in turnover rates. These combinatorial genetic experiments led to the identification of two key substitutions (T117R/G188L) that when made alone in the wt castor Δ^9 -18:0-ACP desaturase background yielded a Δ^9 -16:0-ACP desaturase with a turnover rate approaching that of wt enzyme with 18:0-ACP. This improvement resulted from an increase in k_{cat} and decrease in K_m that yielded an improvement in specificity factor for 16-carbon substrate of 82-fold with respect to wt enzyme.

EXPERIMENTAL PROCEDURES

Random Point Mutagenesis—The portion of the castor Δ^9 -18:0-ACP desaturase corresponding to the mature polypeptide was excised from the clone pRCD1 (3) with the use of *EcoRI* and *XbaI*. The 1683-base pair fragment was subject to partial digestion with DNase I (Sigma) and the resulting fragments separated by agarose gel electrophoresis. Fragments ranging in size between 100 and 250 base pairs were excised and subjected to primerless PCR to facilitate assembly of the full-sized gene along with the incorporating point mutations (23). The authentic sized product was excised from an agarose gel and amplified by PCR using primers: 5'-CACACAGTCTAGAAAATAATTTTGTTAACCTTAAGAAGGA-3' (Primer A) and 5'-GTCTTCAAGAATTCTCATGTTTGACAGCTATATCATCG-3' (Primer B). The amplified fragment was restricted with *XbaI* and *EcoRI* and cloned into the corresponding sites of pLac3. The resulting library of mutants was transformed into *E. coli* MH13/pAnFd and the cells challenged to growth in the absence of unsaturated fatty acids.

Saturation Mutagenesis at Codons 117 and 181—Saturation mutagenesis was performed at codons for amino acids 117 and 181 of the castor Δ^9 -18:0-ACP desaturase by replacing the target codon by NNN with the use of overlap-extension PCR (24) as described previously (19). The mature castor Δ^9 -18:0-ACP desaturase was used as the template, and amplification reactions were conducted with *Pfu* polymerase (Stratagene). Saturating mutations were introduced at the codon for amino acid 117 by initially conducting two separate amplification reactions using the following oligonucleotide primer combinations: Reaction 1-Primer A and 5'-CCAAATTGCCAAGACGTCCGACTTGCACCTGTTCATCCCGAACTCCATCCAANNATTCAGCATTGTTG-3' (Primer C) and Reaction 2-5'-GAAACAGGTGCAAGTCCGACGCTTTGGC-

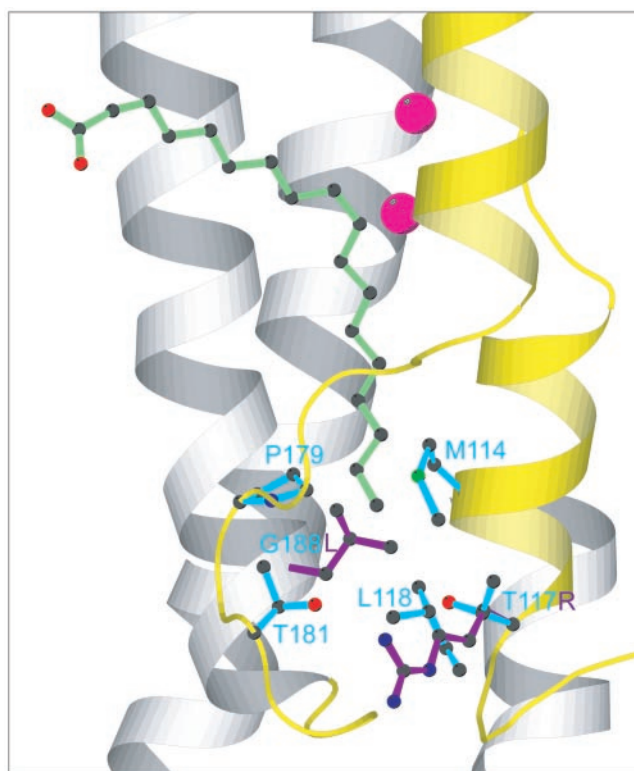


FIG. 1. Location of amino acids selected for combinatorial saturation mutagenesis. A stearic acid moiety shown in green is modeled into the active site, iron ions are shown in magenta, helices and loops are shown (gray behind the plane of the fatty acid and yellow in front). The six residues subjected to mutagenesis are indicated in cyan, and the modeled T117R/G188L substitutions in mutant 5.2 are shown in violet. Atom colors: gray, carbon; blue, nitrogen; red, oxygen; and green, sulfur.

AA-3' (Primer D) and 5'-GTTTCTGTCCGCGGATCCATTCCTG3' (Primer E). Agarose gel-purified reaction products were combined and amplified together with primer A and Primer E. The resulting product was digested with *XbaI* and *SacII* and ligated in place of the corresponding portion of the coding sequence for the mature wild-type castor Δ^9 -18:0-ACP desaturase in pLac3d. Saturation mutagenesis at codon 181 was achieved by amplification of the castor desaturase with: 5'-GGTTCAGGAATGATCCGCGGNNNGAAAAGAGTCCATACC-3' (Primer F) and 5'-GCAAAAGCCAAAACGGTACCATCAGGATCA-3' (Primer G). The resulting fragment was restricted with *BamHI* and *Acc65I* and cloned into the corresponding portion of the coding sequence for the mature wild-type castor Δ^9 -18:0-ACP desaturase in pLac3d.

Combinatorial Saturation Mutagenesis—Overlap extension PCR was used to construct a library in which combinations of one of 20 possible amino acids were expressed at each of six positions (114, 117, 118, 179, 181, and 188) of the castor Δ^9 -18:0-ACP desaturase simultaneously. The codon NNK(G/T) was introduced at each of these targeted positions, because it has 32-fold degeneracy rather than the 64-fold degeneracy of NNN, and it encodes all 20 amino acids without rare codons. Three independent PCR amplification reactions of the wild-type castor Δ^9 -18:0-ACP desaturase were performed using the following oligonucleotide primer combinations: Reaction 1-Primer A and 5'-TTG-

TABLE II
Substitutions at specific positions of the castor Δ^9 -18:0-ACP desaturase mutants containing the T117R/G188L pair of mutations and their specific activity relative to wt

	Position ^a						Relative activity ^b , chain length	
	114	117	118	179	181	188	14	16
Wt	M	T	L	P	T	G	1	1
com2	A	R	G	V	V	L	52	15
com3	Q	R	P	V	D	L	0	1
com4	T	R	A	L	S	L	16	11
com9	V	R	G	S	C	L	3	4
com10	Y	R	P	A	F	L	3	2
5.2 ^c	M	R	L	P	T	L	24	35

^a Saturation mutagenesis was performed at all six sites simultaneously, see "Experimental Procedures."

^b See Table I for specific activity information.

^c Mutant 5.2, T117R/G118L in wt background.

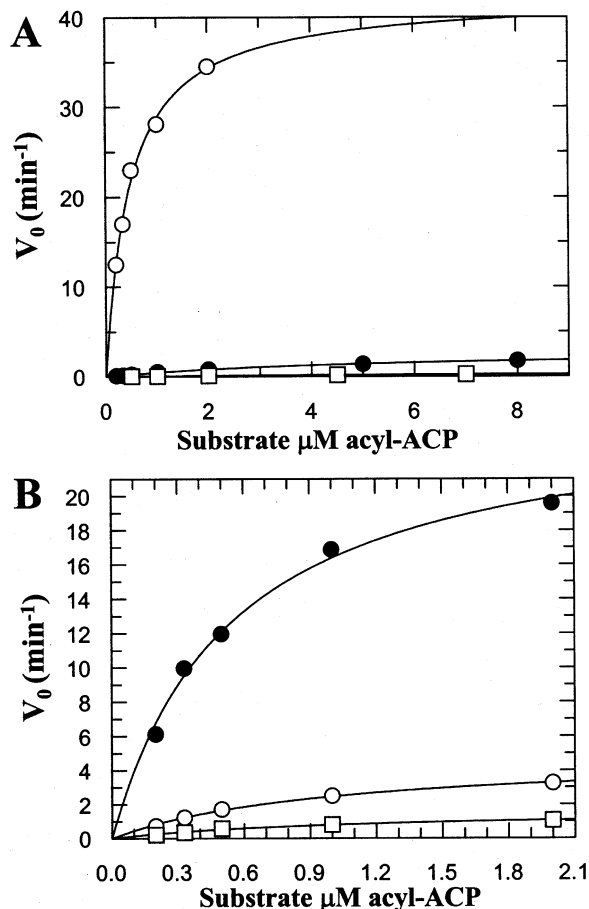


FIG. 2. Plots of initial velocity versus substrate concentration for castor Δ^9 -18:0-ACP desaturase (A) and mutant 5.2, containing T117R/G188L in wt background (B). Curves for three substrates are shown: 18:0-ACP, open circles; 16:0-ACP, solid circles; and 14:0-ACP, open squares. Each point represents the mean of three experiments. See Table III for a summary of kinetic parameters and associated errors.

ATAAGTGGGAAGGGCTTCTCCGTT-3' (Primer H), Reaction 2-5'-AACGGAAGAAGCCCTTCCCACTTATCAAACANNKCTGAATNN-KNNKGATGGAGTTCGGGATGAAAC-3' (Primer I), and 5'-TCCATT-CCTGAACCAATCAAATATTG-3' (Primer J), Reaction 3-5'-TTGATT-GGTTCCAGGAATGGATNNKCGNNKGAAAACAGTCCATACCTT-NNKTTTCATCTATACATCATTC-3' and Primer G. The three agarose gel-purified reaction products were combined and amplified together with Primer A and Primer G. The resulting product was digested with *Xba*I and *Acc*65I and ligated in place of the corresponding portion of the coding sequence for the mature wild-type castor Δ^9 -18:0-ACP DES in pLac3d. The library contained $>10^6$ independent clones. A sample of 19 desaturase clones isolated from colonies that exhibited strong growth in the complementation assay were sequenced. All showed unique sequence combinations at the six targeted positions.

Construction of Mutant 5.2 (T117R/G188L) of the Castor Δ^9 -18:0-ACP Desaturase—Mutant 5.2 was constructed by ligation of the *Kpn*I-*Bam*HI fragment from mutant T117R into the corresponding region of mutant G188L.

***E. coli* Unsaturated Fatty Acid Auxotroph Complementation**—Castor Δ^9 -18:0-ACP desaturase variants in plasmid pLac3d were transformed into *E. coli* MH13/pAnFd cells (19). Cells were grown on LB plates that contained ampicillin (100 $\mu\text{g}/\text{ml}$), chloramphenicol (35 $\mu\text{g}/\text{ml}$), and kanamycin (40 $\mu\text{g}/\text{ml}$) (for selection of pLac-des, pLacAnFd, and the MH13 strain, respectively). For permissive growth, plates were supplemented with oleic acid and Tergitol NP-40 (Sigma) at final concentrations of 250 $\mu\text{g}/\text{ml}$ and 0.2% (v/v), respectively. Selection for complementation was performed on agar plates containing 0.4 mM isopropyl- β -D-thiogalactopyranoside (lacking unsaturated fatty acid) incubated at 30 $^\circ\text{C}$. Complementation was confirmed by growth of selected colonies in liquid media of the same composition at the same temperature. The sequences of the selected variants of the castor Δ^9 -18:0-ACP desaturase were determined to identify the substitution(s).

Fatty Acid Analyses—MH13/pAnFd cells expressing castor acyl-ACP desaturase variants in vector pLac3 were grown at 30 $^\circ\text{C}$ to $A_{600} \approx 0.3$ to 0.5 in 25 ml of LB media supplemented with chloramphenicol, kanamycin, ampicillin, and isopropyl- β -D-thiogalactopyranoside as described above. Cells were collected by centrifugation and lipids extracted and dried under N_2 (25). Fatty acid methyl esters were made by transesterification with sodium methoxide (26) and analyzed with the use of a Hewlett-Packard 6890 GC fitted with a 30 m \times 0.25 mm (inner diameter) HP-INNOWAX (Hewlett-Packard) column. Oven temperature was raised from 170 $^\circ\text{C}$ at 3.5 $^\circ\text{C}/\text{min}$ to 185 $^\circ\text{C}$ after a 25-min hold. Double bond positions of monounsaturated fatty acid methyl esters were located by gas chromatography-mass spectrometry of dimethyl disulfide derivatives (27).

Substrate Specificity and Kinetic Analysis of Castor Δ^9 -18:0-ACP Desaturase Variants—Desaturase variant open reading frames were transferred from pLac3d to pET9d. Recombinant protein was generated by expression in *E. coli* BL21(DE3) cells and enriched to 90–95% purity by 20CM cation exchange chromatography (Applied Biosystems). Castor Δ^9 -18:0-ACP desaturase variants were assayed with [^{14}C]14:0-, [^{14}C]16:0-, or [^{14}C]18:0-ACP substrates with the use of recombinant spinach ACP-I (28). Methyl esters of fatty acids were analyzed by argentation TLC and radioactivity in products quantified as described previously (11, 18). Wild type castor Δ^9 -18:0-ACP desaturase and mutant 5.2 assays were performed in triplicate for each of five concentrations typically ranging from one-fifth to five times the K_m (where practical) and with each of three substrates: 14:0-, 16:0- and 18:0-ACP. Data were processed with the use of the GraFit data analysis and graphics suite version 3.01 (29). Kinetic parameters and statistics were based on preprogrammed algorithms within the GraFit suite.

RESULTS

Choice of Residues for Mutagenesis—It is difficult to predict the locations of amino acids that can affect a property of interest (30). For this reason we performed random mutagenesis of the entire 363-amino acid coding region of the desaturase and subjected the mutant population to a selection system to identify mutants active on substrates with chain length <18 carbons. In this way, five positions (114,⁴ 117, 118, 179, and 181) capable of affecting chain length specificity were identified (Table I). Mutagenesis was at, or close to, saturation based on

TABLE III
Kinetic parameters of the wt castor Δ^9 -18:0-ACP desaturase and mutant 5.2

Substrate chain length	wt			5.2 ^a		
	k_{cat}^b	K_m	Specificity factor k_{cat}/K_m	k_{cat}	K_m	Specificity factor k_{cat}/K_m
	min^{-1}	μM	$\mu\text{mol}^{-1} \cdot \text{min}^{-1}$	min^{-1}	μM	$\mu\text{mol}^{-1} \cdot \text{min}^{-1}$
18	42.3 (1.6)	0.46 (0.05)	92.0	4.9 (0.25)	0.98 (0.10)	5.0
16	2.8 (0.12)	5.00 (0.43)	0.56	25.3 (1.1)	0.55 (0.06)	46.0
14	0.39 (0.03)	4.64 (0.79)	0.08	1.64 (0.17)	1.06 (0.22)	1.55

^a See Table II.

^b k_{cat} is reported per diiron site.

^c Mean with S.E. in parentheses.

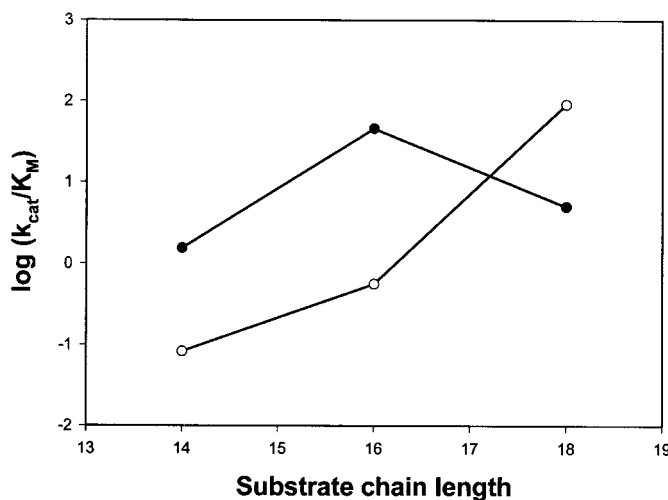


FIG. 3. Relationship between $\log(k_{\text{cat}}/K_m)$ and chain length of the acyl-ACP substrate for wt castor desaturase (open circles) and mutant 5.2 (closed circles). The data are discrete, thus lines are drawn solely for ease of visualization.

the isolation of the same amino acid changes multiple times (data not shown). Three of the amino acid positions (114, 118, and 179) identified by random mutagenesis were coincident with a group of four (114, 118, 179, and 188) that were previously shown to affect chain length specificity (19), and two new amino acid locations, 117 and 181, were identified (Fig. 1). In the case of this soluble desaturase enzyme, all six amino acid positions cluster to the region adjacent to the bottom of the substrate-binding pocket, and no sites remote from the substrate binding site were identified. These six locations were therefore selected as targets for combinatorial mutagenesis.

Comparison of the activities of point mutants at each of the selected sites with those from saturation mutagenesis at the same location showed that for four of the six sites (114, 117, 179, and 181) superior mutants were obtained by saturation mutagenesis, an equal improvement for the fifth site (118), and no point mutation was obtained for the sixth site (188) (Table D). The observation that saturation mutagenesis at single sites resulted in larger improvement in the property of interest compared with point mutagenesis prompted us to ask if even larger improvements in specific activity for substrates with fewer than 18 carbons could be made by simultaneously randomizing the six positions able to affect substrate specificity.

Combinatorial Saturation Mutagenesis—A library of variants randomized for amino acid substitutions at all six sites was constructed by overlap extension PCR. To select for improvement in activity with substrates <18 carbons in length, the library was introduced into the unsaturated fatty acid auxotroph and challenged on media lacking unsaturated fatty acids.

Nineteen colonies that exhibited strong growth under selective conditions subjected to both sequence and activity analy-

ses. Sequence analysis revealed that all 19 clones contained a unique combination of amino acids at the six randomized positions. When analyzed for specific activity, com2 (M114A/T117R/L118G/P179V/T181V/G188L) showed the greatest improvement in substrates containing fewer than 18-carbons, *i.e.* 52-fold for 14-carbon substrates and 15-fold for 16-carbon substrates (Table II). Of note, five of the 19 strongly complementing mutants (com2, com3, com4, com9, and com10) contained the same pair of changes, T117R/G188L, found in com2 (Table II). The wide range of specific activities among the different complementing mutants containing the T117R/G188L pair of mutations suggested the presence of detrimental mutations in one or more of the remaining four positions. This was confirmed by construction of the double mutant (5.2) T117R/G188L (in which the other targeted positions contained residues found in wt desaturase) that showed a 35-fold increase in specific activity with respect to the 16-carbon substrate and a 24-fold increase for the 14-carbon substrate (Table II).

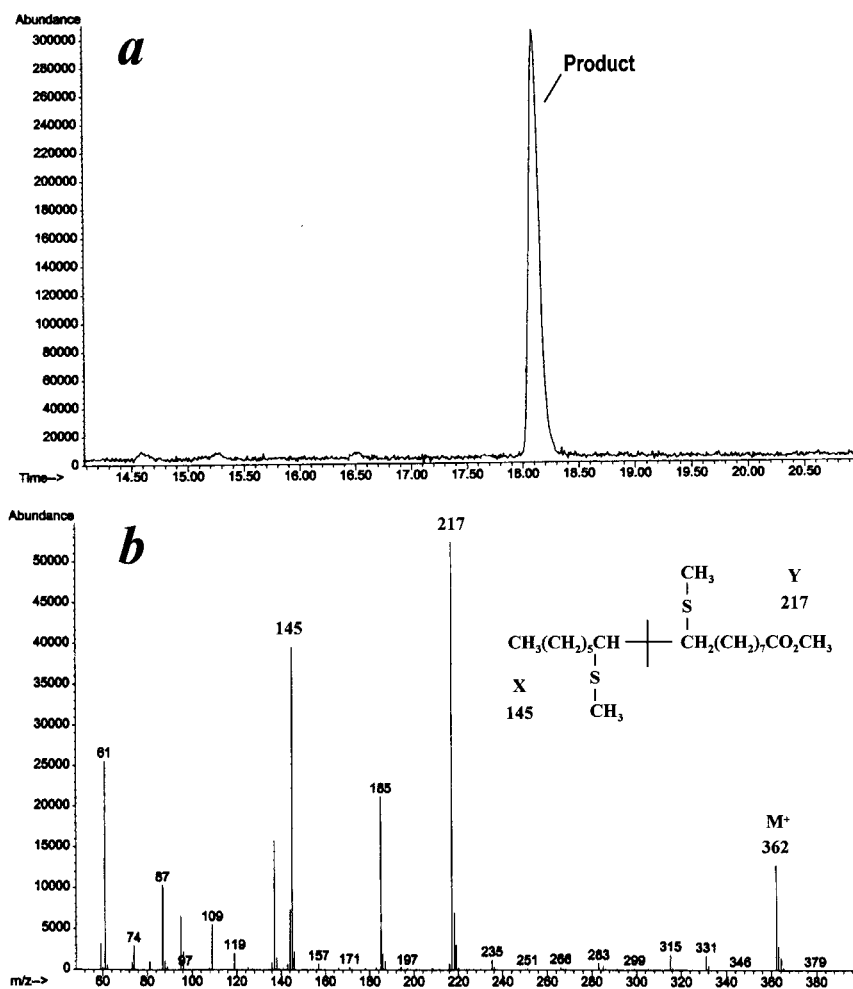
Kinetic Analysis of wt and Mutant 5.2—To gain insight into the mechanism underlying changes in substrate specificity, we performed a comparative kinetic analysis on wt and mutant 5.2 (Fig. 2 and Table III). The data show that wt desaturase has a different K_m for different substrates, 0.46 μM for 18-carbon, 5.0 μM for 16-carbon, and 4.6 μM for 14-carbon substrates. However, in the case of the double mutant 5.2 the K_m for 16-carbon substrate has decreased by an order of magnitude from 5.0 to 0.55 μM , while the K_m for 18- and 14-carbon substrates have changed from 0.46 to 0.98 μM and from 4.6 to 1.1 μM , respectively. Together these changes result in an increase in specificity factor from 0.56 to 46 $\mu\text{mol}^{-1} \text{min}^{-1}$ for the 16-carbon substrate, and a decrease from 92 to 5 $\mu\text{mol}^{-1} \text{min}^{-1}$ for the 18-carbon substrate (Table III). A graphical representation of the specificity data for wt and mutant 5.2 is presented in Fig. 3.

Regiospecificity—Selection for one parameter can lead to undesirable changes in unrelated parameters (31). For the desaturase changes in regiospecificity had accompanied earlier attempts to alter chain length specificity (19). We therefore determined the position of the introduced double bond by gas chromatography-mass spectrometry analysis of the dimethyl disulfide derivative of the desaturated fatty acid product of mutant 5.2. The only detectable product was 16:1 in which the double bond was exclusively located at the Δ^9 -position (Fig. 4) showing that the regiospecificity is unchanged from that of wt desaturase.

DISCUSSION

Combinatorial saturation mutagenesis of six residues shown individually to affect chain length specificity, in combination with selection using an unsaturated fatty acid auxotrophy complementation system in combination with logical redesign, led to the engineering of a desaturase enzyme with preference for 16-carbon substrate with kinetic parameters similar to those of wt desaturase for the 18-carbon substrate. The activity of the resulting mutant 5.2 is >100-fold improved with respect to those reported for naturally occurring 16:0- Δ^9 desaturases

FIG. 4. Gas chromatographic analysis of dimethyl disulfide-derivitized methyl esters of the reaction product of mutant 5.2 with 16:0-ACP substrate (a) and mass spectrometric analysis of the product peak (b). The source of the molecular ion M^+ and fragments X and Y are indicated.



from Milkweed and *Doxantha* (5, 11).

To understand the mechanism underlying the observed improvement in specific activity of mutant 5.2, we performed a kinetic analysis in which we included wt castor Δ^9 -18:0-ACP desaturase as the control. The experiments with wt desaturase yielded a K_m value for 18-carbon substrate of $0.46 (\pm 0.05) \mu\text{M}$. In contrast, previous studies have reported a range of K_m values: $3.3 (\pm 0.42) \mu\text{M}$ for (the same castor desaturase) (7), $0.38 \mu\text{M}$ for the safflower enzyme (4), and $13 (\pm 8) \mu\text{M}$ for a soybean isoform (32). We have confidence in the values reported here for several reasons. First, the estimates of substrate concentrations are based on specific radioactivity of the substrate; second, the values show the smallest statistical variability; and third, the values are based on a ranges of substrate concentrations typically from one-fifth to five times the K_m value. All of these conditions were not met in the studies described above. In addition, the values for wt enzyme presented here are consistent with the pool sizes of long chain acyl-ACPs in plants that are in the high nM range (33), *i.e.* comparable with the K_m for 18:0-ACP, but below the K_m for 16:0-ACP.

The K_m value for mutant 5.2 with the 16-carbon substrate decreased ~ 10 -fold with respect to that of the wt enzyme, *i.e.* to $0.55 (\pm 0.06) \mu\text{M}$ from $5.0 (\pm 0.56) \mu\text{M}$, respectively. It is interesting to note that the physiological selection system used to identify the mutations used to engineer mutant 5.2 resulted in an enzyme with a K_m value almost identical to the K_m of $0.46 (\pm 0.06) \mu\text{M}$ reported (above) for the wt enzyme with the 18-carbon substrate.

In addition to changes in binding affinities for different substrates between wt and mutant 5.2, there were also substantial

changes in k_{cat} . Mutant 5.2 showed a large increase in k_{cat} of 25.3 min^{-1} as compared with 2.8 min^{-1} for wt enzyme with the 16-carbon substrate. Together, these changes in K_m and k_{cat} resulted in an 82-fold increase in the specificity factor (k_{cat}/K_m) for 16-carbon substrates with mutant 5.2, accompanied by an 18-fold decrease for 18-carbon substrates (Table III). The decrease in $\log(k_{\text{cat}}/K_m)$ for wt desaturase as the substrate length decreases from 18 to 14 carbons (Fig. 3) is similar to that reported previously (7). However, the differences in both K_M and k_{cat} values between wt and mutant 5.2 suggest the factors that contribute to turnover may be more complex than simply the change in binding energy as proposed previously for wt enzyme with different substrates (7). Thus for mutant 5.2, the decrease in binding energy of the 16-carbon substrate did not prevent the observed 35-fold improvement in specific activity.

In structural terms the change in specificity factor between 18- and 16-carbon substrates likely represents loss of methyl contact at the base of the binding pocket in addition to two methylenes from the wall of the pocket. This has a larger effect on affinity and/or substrate positioning than the loss of interaction of a further two methylenes with the wall of the binding pocket with a 14-carbon substrate. In mutant 5.2, bulkier arginine and leucine residues replace threonine and glycine at the end of the binding pocket, apparently shortening the binding pocket and making it more effective for a 16-carbon substrate but less effective for an 18-carbon substrate. The com2 and com4 enzymes on the other hand are more active on 14- than 16-carbon substrates, presumably reflecting the contributions of other residues to binding affinity or positioning of the substrate for catalysis.

Powerful methods have recently been developed to evolve enzymes *in vitro* (34). They generally use point mutagenesis and identify improved variants followed by recombination of improved isolates to effect further improvements in the property of interest. A limitation inherent to this approach is that it relies on the relatively small subset of four to seven mutations achievable by single base substitution at each location. We note that neither of the pair of substitutions identified in this study, T117R and G188L, would have been reached by single-base substitution. These results support the contention that saturation mutagenesis can generate superior variants to those arising from the subset of possibilities available via point mutagenesis (35). In this context, amino acids can be regarded as “molecular shims,” and the larger the number of shim sizes introduced at a particular amino acid location, the more likely that some “optimal substrate binding geometry” will be approached (36). Performing simultaneous saturation mutagenesis at a number of key locations capable of affecting the property of interest simply extends the number of combinations and permutations of binding site geometry. In this case, the loss of desolvation energy of two methylene groups when binding a 16- rather than an 18-carbon substrate was mostly overcome by improved substrate binding orientation with respect to the active site oxidant and improved substrate fit resulting from occlusion of the floor of the binding pocket.

In summary, these data show that it is possible to alter the specificity of a desaturase enzyme without substantial degradation of its kinetic parameters. The Δ^9 -16:0-ACP desaturase, 5.2, constructed from a Δ^9 -18:0-ACP desaturase is far more active than either naturally occurring Δ^9 -16:0-ACP desaturases. Taken together, these results suggest it should be generally possible to reengineer archetypal enzymes with substrate specificities characteristic of newly evolved enzymes while retaining the desired stability and/or turnover characteristics of the parental paralog.

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