The Crystal Structure of the Ivy Δ^4-16:0-ACP Desaturase Reveals Structural Details of the Oxidized Active Site and Potential Determinants of Regioselectivity*

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The multifunctional acyl-acyl carrier protein (ACP) desaturase from Hedera helix (English ivy) catalyzes the Δ^4 desaturation of 16:0-ACP and the Δ^9 desaturation of 18:0-ACP and further desaturates Δ^5-16:1 or Δ^2-18:1 to the corresponding Δ^4,Δ^9 dienes. The crystal structure of the enzyme has been solved to 1.95 Å resolution, and both the iron-iron distance of ~3.2 Å and the presence of a μ-oxo bridge reveal this to be the only reported structure of a desaturase in the oxidized FeIII-FeIII form. Significant differences are seen between the oxidized active site and the reduced active site of the Ricinus communis (castor) desaturase; His^227 coordination to Fe2 is lost, and the side chain of Glu^224, which bridges the two iron ions in the reduced structure, does not interact with either iron. Although carboxylate shifts have been observed on oxidation of other diiron proteins, this is the first example of the residue moving beyond the coordination range of both iron ions. Comparison of the ivy and castor structures reveal surface amino acids close to the annulus of the substrate-binding cavity and others lining the lower portion of the cavity that are potential determinants of their distinct substrate specificities. We propose a hypothesis that differences in side chain packing explains the apparent paradox that several residues lining the lower portion of the cavity in the ivy desaturase are bulkier than their equivalents in the castor enzyme despite the necessity for the ivy enzyme to accommodate three more carbons beyond the diiron site.

Fatty acid acyl carrier protein (ACP)^3 desaturases (EC 1.14.99.6) convert saturated fatty acyl-ACPs into their cis-mono-unsaturated equivalents in an oxygen-dependent reaction (1–6). Δ^9-desaturation of 18:0-ACP^4 occurs ubiquitously in the plastids of plants and thus plays an important role in determining the fluidity of cell membranes (7). Plants can express distinct acyl ACP desaturases, e.g. Arabidopsis thaliana has a family of seven acyl-ACP desaturases that are differentially expressed in different tissues (8). A structurally related homolog with unknown function from Mycobacterium has also been described (9).

Seed oils of various plants accumulate monounsaturated fatty acids with unusual chain lengths and/or double bond positions. Several Δ^9 desaturases that recognize 14-, 16-, and 18-carbon chain lengths (4, 10–12) have been described, and others with Δ^6 and Δ^9 regiospecificities have also been reported (13, 14). The biosynthetic origin of unusual fatty acids is not always straightforward; for instance, Coriandrum sativum L. (coriander) and Hedera helix (English ivy) accumulate petersilicin acid (18:1Δ^6) that originates via a Δ^4 desaturation of 16:0-ACP followed by 2-carbon elongation to 18:1Δ^6 (15). The genes encoding these Δ^6-16:0-ACP desaturase enzymes have been isolated (15, 16), and their amino acid sequences are homologous to that of the castor Δ^3-18:0-ACP desaturase for which the crystal structure has been determined (17, 18). This implies that acyl-ACP desaturases share a common architecture that is able to accommodate different substrate binding modes, which leads to the observed desaturation of fatty acids of different chain lengths and/or at different position along the fatty acid. However, desaturase structures with substrate in the active site are yet to be reported.

Several of these desaturases have been used in experiments designed to identify the determinants of desaturase specificity. The study of chimeras of the ~360-amino acid sequences of the mature castor Δ^3-18:0- and the Thunbergia Δ^3-16:0-ACP desaturases and information from the crystal structure of the castor Δ^9-18:0-ACP desaturase led to the identification of five residues in the castor sequence that, when substituted into the corresponding positions in the Thunbergia sequence, con-

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* This work was supported in part by funds from the Office of Basic Energy Sciences of the United States Department of Energy (to J. S.) and from Swedish Foundation for International Cooperation in Research and Higher Education and the Swedish Research Council (to Y. L.). This work was also supported in part by funds from the Offices of Biological and Environmental Research and of Basic Energy Sciences of the U. S. Department of Energy (to the National Synchrotron Light Source) and from the National Center for Research Resources of the National Institutes of Health. 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.

** The on-line version of this article (available at http://www.jbc.org) contains supplemental Fig. S1.

The atomic coordinates and structure factors (code 2uw1) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org).

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3 The abbreviations used are: ACP, acyl carrier protein; MES, 4-morpholineethanesulfonic acid; MMO, methane monooxygenase.
4 Fatty acid nomenclature: x:y, x is the number of carbon atoms in the fatty acid chain, and y is the number of double bonds. Δ^n indicates the regiospecificity, i.e., the position of the double bond relative to the carboxyl end of the fatty acid.
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verted the Thunbergia Δ⁶-16:0-ACP desaturase into Δ⁶-18:0-ACP desaturase (19). However, subsequent attempts to resolve which residues contribute to chain length specificity and which to regioselectivity were unsuccessful. Mutagenesis selection experiments on the castor enzyme revealed individual determinants of chain length specificity (20), but the determinants of regioselectivity remain elusive.

The ivy desaturase introduces a double bond five carbons closer to the carboxyl end of the fatty acid compared with the castor enzyme, with which it shares 74% amino acid identity. In the crystal structure of the castor enzyme, each monomer comprising the castor enzyme homodimer has a boomerang-shaped cavity adjacent to the active site diiron cluster. Modeling the substrate in an extended conformation into the cavity suggests that when the methyl group of the steareate is in contact with the bottom of the cavity, C-9 and C-10 are positioned with their pro-R hydrogens facing the diiron active site consistent with the introduction of a cis Δ⁶ double bond (17) and the Pro-R, Pro-R, stereochemistry reported for castor desaturation (21). This model is also consistent with the location of the azide in the desaturase-azide complex, which mimics the active site oxidant responsible for hydrogen abstraction (18).

It is remarkable that desaturase enzymes are able to introduce double bonds between specific carbons of the fatty acid substrate with >99% fidelity because saturated acyl chains are composed of a series of methylene groups; the nearest potential recognition elements being the methyl, or thioester groups, each 9 carbons distant from the site of double bond formation in the castor enzyme. Because the ivy desaturase introduces a Δ⁴ double bond into a 16:0-substrate, five carbons distant from that of the castor desaturase with the equivalent substrate, and with the same Pro-R Pro-R stereoselectivity as the castor enzyme (22), we reason that differences in substrate binding between the two enzymes must account for differences in observed regioselectivity. We have initiated x-ray crystallographic analysis of the ivy desaturase to address this hypothesis. Here we present a crystal structure of the ivy desaturase at 1.95 Å resolution and identify several candidate regioselectivity determinants. The structure contains the first report of an oxidized (i.e. resting FeIII-FeIII) diiron active site in a desaturase. Comparison of this structure with that of the reduced castor desaturase reveals that the side chain of Glu²²⁴, which bridges the two iron ions in the reduced state, undergoes a large carboxylate shift upon oxidation, completely disengaging from the iron ions.

**EXPERIMENTAL PROCEDURES**

*Construction and Production of Three Ivy Desaturase Constructs*—The ivy desaturase constructs engineered by PCR, and Ndel and BglII restriction sites were introduced at the 5’ and 3’ ends of the genes, respectively, that were used to clone the desaturase sequence into the Ndel and BamHI sites of pET9a. Ivy1 was amplified with the following primers: ivycrnf, 5’-GAATTCGGGCTCAAGAATTACCTCTTAATTGACACCC-3’, and ivy2r, 5’-GTGACTAGATTTCTACATATCTTC-3’. Ivy1 was sequenced to ensure the clones were free from secondary mutations. Expression and purification of the ivy desaturase constructs were performed as previously described for the full-length mature ivy desaturase (16).

**Crystallization and Data Collection**—Prior to crystallization, the ivy desaturase corresponding to construct ivy2, which is truncated at its N terminus and has the C terminus of the castor desaturase, was concentrated to a final protein concentration of 10 mg ml⁻¹ in 20 mM Tris, pH 7.0, and 100 mM NaCl. Crystallization was performed by the hanging drop vapor diffusion method, using a precipitant solution of 25% (w/v) polyethylene glycol MME 550 and 0.1 M MES buffer, pH 6.6. Drops containing 5 μl of protein solution mixed with 1 μl of the precipitant solution, 1 μl of 30% glycerol, and 0.5 μl of 0.25 M potassium fluoride were allowed to equilibrate at 7 °C. Diffraction quality bipyramidal crystals grew under these conditions after ~2 days.

The data were collected to a resolution of 1.95 Å at cryogenic temperatures on Beamline X25 of the National Synchrotron Light Source (Brookhaven, NY). The crystals were cryoprotected in 25% glycerol and flash-frozen directly in the nitrogen stream. All of the data used to solve and refine the structure were collected from a single crystal.

Data processing and scaling were performed using the HKL2000 software suite (24). The scaled intensities were then converted to CCP4 format using Scalepack2MTZ from the CCP4 suite (CCP4, 1994), and structure factors were calculated from the intensities with TRUNCATE.

**Structure Determination and Refinement**—The structure of the ivy desaturase was solved by molecular replacement as implemented in the program MOLREP (25), using a monomer of the castor Δ⁹ desaturase structure (17) Protein Data Bank accession code 1AFR) as the search model. After initial rigid body refinement in REFMAC5 (26), the ivy desaturase sequence was manually built into the model using the program COOT (27). The model was improved by manual inspection and rebuilding in COOT and refined using the maximum likelihood method implemented in REFMAC5 (26). Atomic displacement parameters were refined in REFMAC by the TLS (translation, libration, screw) method, with each of the two monomers in the asymmetric unit treated as a single TLS group. All refinement excluded the 5% of reflections set aside for R_free calculations. Water molecules were automatically assigned to F_obs – F_c difference density peaks in COOT and were subsequently inspected manually using both 2F_obs – F_c and F_obs – F_c maps. Annealed composite omit maps were calculated with...
the use of the Crystallography and NMR System (CNS) software suite (28) and were used to check the accuracy of the final model.

Structure Analysis—The geometry of the refined structure was analyzed with PROCHECK (29), and the fit between the data and model was assessed in SFCHECK. A summary of the statistics is given in Table 1. Structure alignments were carried out using the SSM superposition function in COOT and with the LSQ option in the program O (30) using default parameters. All figures of the structure were produced using PyMol (31). The structure factors and the coordinates of the final model have been deposited in the Protein Data Bank with accession code 2uw1.

RESULTS AND DISCUSSION

Cloning, Purification, and Crystallization—A goal of this study was to obtain a high resolution crystal structure of the ivy \(/H_9004\) 4-16:0-acyl carrier protein desaturase and to compare it with that of the previously reported castor \(/H_9004\) 9-18:0-ACP desaturase to identify potential determinants of regioselectivity. Initial attempts at obtaining crystals with a mature ivy desaturase construct described previously (16) were unsuccessful. We therefore engineered three new constructs with modifications at the N and C termini. Because density for the N-terminal 16 amino acids is not seen in the castor desaturase crystal structure (17), we engineered an ivy desaturase in which the first 20 residues of the mature open reading frame \((\text{i.e. beyond the transit peptide cleavage site})\) were replaced by a single initiating methionine to create ivy1. The ivy and castor desaturases show strong divergence at their C termini. The ivy sequence contains several lysine residues not found in the castor sequence. To determine whether these C-terminal differences were preventing the ivy desaturase from forming crystals, we engineered ivy3, in which residues 322–360 were replaced by the equivalent 38-residue portion of the castor desaturase. The N- and C-terminal changes of ivy1 and ivy3 were combined to produce ivy2, which is truncated at its N terminus and has the C terminus of the castor desaturase.

Of the three new ivy constructs, only ivy2 produced diffraction quality crystals. Because the study is designed to address the determinants of regioselectivity and ivy2 is modified at its N and C termini with respect to the mature ivy desaturase sequence, it was critical to determine whether the changes made to obtain crystals had affected the specificity of the desaturation reaction. We therefore performed assays with mass labeled, \(\text{i.e. deuterated} (7,8D_4) 16:0-\text{ACP}, \) such that the desaturation products could be unambiguously distinguished from low levels of background fatty acids originating from the reaction components. These experiments show that the fatty acid methyl ester of the reaction product has a gas chromatographic retention time characteristic of a 16:1\(^4\) product (Fig. 1, \(A\) and \(B\)). Analysis of DMDS derivatized fatty acid methyl esters

![Figure 1](image-url)
of the reaction products showed ivy2 to desaturate 16:0 exclusively at the Δ^4-position (Fig. 1C) as reported for the mature ivy enzyme (16). In addition to characterizing the regioselectivity of the ivy2 chimeric desaturase, we also determined its kinetic parameters (Table 2). No significant differences were observed for k_cat, K_m, or specificity factor k_cat/K_m between the ivy2 and the wild type ivy desaturase. We conclude that neither the N-terminal 20 amino acids nor the identity of the C-terminal 38 amino acids influence either the specificity of desaturation or its kinetic properties, and the following structural data regarding regioselectivity and details of the oxidized active site for ivy2 therefore accurately represents that of the wild type ivy desaturase.

Diffraction quality crystals of the ivy desaturase grew in ~2 days, reaching maximum dimensions of 0.2 × 0.15 × 0.15 mm. The crystals belong to the orthorhombic space group P2_12_1 with cell dimensions a = 61.12 Å, b = 61.86 Å, c = 201.0 Å. The asymmetric unit contains two monomers, and the solvent content of the crystals has been estimated at 44.4% (32).

Quality of the Electron Density Map and the Model—The ivy desaturase model has been refined to an R factor of 18.4% and an R_free of 22.8%. The resolution of 1.95 Å is significantly higher than previously observed for an acyl-ACP desaturase structure, and the overall quality of the final electron density maps is very good. The asymmetric unit contains the biological dimer, consisting of two desaturase monomers related by a noncrystallographic 2-fold symmetry axis. Despite the high overall similarity of the two monomers, differences, due primarily to crystal packing effects, are seen in some regions of the electron density. No density is seen for the first 13 residues of monomer A, although the entire N terminus is clearly visible in monomer B. Monomer A shows relatively high quality density for residues 337–353, located in a flexible loop region, whereas the equivalent residues in monomer B are very poorly defined and have therefore been modeled with occupancy of zero. The Δ^18:0-ACP desaturase from Ricinus communis (castor) appeared to be highly flexible in previous structural studies, with high B-factors observed throughout the molecule and particularly in surface loops. Although the current structure shows the same loops to be the most flexible regions of the protein, the average B-factor of 26.0 is significantly lower, suggesting a higher level of order in the crystals.

A total of 555 ordered water molecules were included in the structure, and the final model contains 8 residues for which alternative side chain conformations were added. The most favorable regions of the Ramachandran plot as determined by PROCHECK contain 92.6% of the nonglycine residues in the final model. We note that one residue from each subunit, Lys^257, is located in the disallowed region, but this residue is well defined in the electron density. Although Lys^257 is on the surface of the molecule and distant from the active site, the equivalent residue in the castor desaturase was also an outlier in the Ramachandran plot, possibly suggesting a functional role for this conformation. Its positioning close to opening to the substrate-binding channel raises the possibility that it interacts with ACP or substrate.

Overall Structure—The monomer structure of the ivy desaturase is a compact single domain with predominantly α-helical secondary structure (Fig. 2). 11 α-helices are present, with nine forming a large helical bundle. Four of these α-helices represent the conserved four-helix bundle of the diiron enzyme family, each helix contributing residues to the active site that lies between them. Only two short β-strands are present in the structure, which form a small β-hairpin at the C terminus of the monomer.

The overall fold of the monomer is very similar to that of the previously determined castor desaturase, with which it shares 74% sequence identity; superimposition of the monomers results in an root mean square deviation of 0.76 Å for 311 Ca atoms (Fig. 3). The superimposed Ca traces differ most significantly in the region of residues 332–342 (ivy numbering), which correspond to the C-terminal end of helix 11 and part of a flexible loop, both of which are on the predicted binding surface for ACP (33).

The quaternary structure of the desaturase is a highly symmetrical dimer (Fig. 2), and monomer–monomer interactions are essentially similar to those seen in the castor desaturase. The monomer–monomer interface buries 5582 Å^2 of solvent-accessible surface, 18% of the total dimer area. During refinement, density for two additional atoms was found in the dimer interface on the 2-fold symmetry axis and is thought to correspond to two sodium ions from the crystallization buffer. One sodium ion binds to the carboxyl oxygen of Glu^108 from subunit A, and the other binds to the corresponding residue of subunit B. Both ions show octahedral geometry by interacting with five water molecules, two of which bridge the two sodium ions. Density for a metal ion was previously found adjacent to the equivalent residue of the castor desaturase (Glu^106) in the apo

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**TABLE 2**

| Form   | k_cat | K_m | Specificity factor k_cat/K_m |
|--------|-------|-----|-----------------------------|
| ivy2   | 16.5 (2.1) | 1.4 (0.3) | 11.7                       |
| ivy^a  | 15.0 (1.3) | 1.5 (0.2) | 10.0                       |

* k_cat is reported per diiron site. The means are based on four or five rates. The numbers in parentheses represent standard errors.
* a Ivy data are from Whittle et al. (16).
desaturase structure (18). In this case strontium had been present in the crystallization medium, and the density was assigned as a single strontium ion with bidentate coordination from Glu106 of each subunit.

The Oxidized Active Site Structure—The most striking feature of the ivy desaturase active site is the oxidized state of the diiron center. Previous studies of the castor desaturase have shown the diiron site in the reduced (FeII-FeII) form, presumably as a result of photochemical reduction in the x-ray beam, and numerous attempts to capture the oxidized diiron site have been unsuccessful. The oxidized diiron center of the ivy desaturase therefore represents the first detailed structural study of a desaturase active site in its FeIII-FeIII resting state.

In the two monomers of the asymmetric unit, the iron-iron distance is 3.15 and 3.25 Å, respectively; clearly indicating the diferric state rather than the diferrous, in which an iron-iron distance of ~4.2 Å is observed. Further evidence of the oxidized state is provided by the presence of a μ-oxo bridge, the density for which is clearly observed (Fig. 4A) in both monomers. Both the shorter iron-iron distance and the μ-oxo bridge have previously been predicted by extensive spectroscopic studies of the castor desaturase active site in its oxidized state. Resonance Ramen spectroscopy (34) showed an Fe(III)-O-Fe(III) unit with an angle of ~123°, close to the 113° seen in the crystal structure. Studies by EXAFS predicted the majority species in the oxidized desaturase to have an iron-iron distance of 3.12 Å and Fe-O distance of 1.8 Å, corresponding to a (μ-oxo)bis(μ-carboxylato)diron(III) center (35). This is confirmed by the crystal structure, with iron-iron distances of 3.15–3.25 Å and iron-oxygen distances from 1.85–1.95 Å.

In addition to the μ-oxo bridge, each iron is coordinated by several of the active site residues. Fe1 coordinates His141 at a distance of 2.3 Å and makes a bi-dentate interaction with the Glu100 side chain at 2.3 and 2.25 Å. Glu138 forms a bridge across the two irons, binding Fe1 at a distance of 2.1 Å and Fe2 at 2.0 Å. Fe2 interacts directly with only two active site residues, the second being the bidentate ligand Glu191, which binds Fe2 with its two side chain oxygen atoms at 2.25 and 2.3 Å, respectively.
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His227 (unless otherwise indicated, amino acid numbering corresponds to the sequence of the mature ivy desaturase protein), which interacts with Fe2 in the reduced active site of the castor desaturase, is not a ligand in the current structure and instead interacts weakly via a water molecule located 2 Å from Fe2 (Fig. 4, compare B and C). The final position in the coordination sphere of each iron is taken by a bidentate small molecule ligand. No substrate or inhibitor was intentionally added at any stage during purification and crystallization of the desaturase, but electron density for a ligand is present in the active site of the enzyme. Because of the high quality of the density (Fig. 5), it was possible to identify the ligand as a short chain fatty acid, with the oxygens of the carboxyl group interacting with the diiron site. A substituent on the third carbon is clearly seen, with the electron density, B-factors and environment all strongly indicating it to be a hydroxyl group. A variety of 3-hydroxy fatty acids are known to be metabolic intermediates in bacteria (36), and it is highly likely that the ligand originates from the expression host Escherichia coli. The quality of the density decreases toward the methyl end of the ligand, making identification of this section more ambiguous. At least one methyl substituent appears to be present on carbon 5, with much weaker density suggesting a second methyl group. The ligand was ultimately modeled as 3-hydroxy 5-dimethyl hexanoic acid, which provides a good fit to the density and B-factors consistent with those of the surrounding residues. The carboxyl group of the bound ligand is consistent with the position of acetate in complex with the castor desaturase (18). In the native unliganded castor desaturase structure, the same position is occupied by a water molecule that weakly coordinates both iron atoms. In the first crystal structure of MMO (37), an exogenous ligand described as acetate was found in the equivalent position and seen to coordinate the diiron site through its carboxyl oxygens in the same manner. In a later structure of the same protein it was absent and was replaced by a water molecule but without any other changes of the diiron center ligands (38). Both of these facts strongly suggest that the presence of the ligand in the current structure does not alter the coordination or the iron geometry and that if it was absent the same position would be likely to be occupied by a water molecule.

Comparison with the Reduced Desaturase Active Site—The oxidized active site seen in the ivy structure corresponds to the resting form of the desaturase, the state in which the enzyme remains until the catalytic cycle is initiated by binding of the substrate, acyl-ACP. Substrate binding favors reduction of the diiron center by ferredoxin to give the FeII-FeII state (39), observed in the castor desaturase crystal structure, which binds and activates molecular oxygen to enable desaturation of the fatty acid. As the newly desaturated fatty acid and water are released, the active site returns to its FeII-FeII resting state, completing the catalytic cycle. The active site residues of the desaturases from castor and ivy are completely conserved, and comparison of the two x-ray structures therefore gives the first detailed insight into the changes that occur in the active site of acyl-ACP desaturases during their catalytic cycle.

The difference in iron coordination between the two structures results primarily from a 1.3 Å shift in the position of Fe2 to give the shorter iron-iron distance of the oxidized active site (Fig. 6) The position of Fe1 changes much less, with a shift of 0.6 Å. In this new position, Fe2 retains the bidentate coordination of Glu191 (Glu196 in castor desaturase) but is no longer within binding distance of His227 (His232 in castor desaturase), and coordination to this residue is lost. In the oxidized structure an additional water molecule, W2, is found in the active site. W2 replaces His227 in the coordination sphere of Fe2, binding at a distance of 2.0 Å, and also interacts with the histidine ND1 at 2.4 Å distance. His227 is the only active site residue that does not change its side chain position between the oxidized and reduced structures; those that retain coordination to Fe1 consistently show movements of ~0.6 Å, and those coordinating Fe2 move up to 1 Å. In both cases this allows the residues to maintain iron coordination at distances equal to or slightly shorter than those in the reduced state, despite the shifted positions of the iron ions.
The most significant change in residue position between the oxidized and reduced active sites is a shift in the side chain of residue Glu224 (Glu229 in castor desaturase). The current structure reveals a large carboxylate shift in the desaturase active site, with the side chain of Glu224 facing away from the diiron center and making no interaction with either iron ion. This is caused by a 1.8 Å shift in C6, which leaves the carboxyl oxygens more than 3 Å from their original positions and with shortest distances to Fe1 and Fe2 of 3.8 and 4.2 Å, respectively. Movement of Glu224 in the oxidized active site effectively creates space for formation of the μ-oxo bridge. The bridging oxygen is positioned on the same side of the diiron center as the shifted carboxylate and interacts with OE2 of the glutamate side chain at a distance of 2.7 Å.

OE2 of Glu224 also interacts with OG1 of Thr194, a residue just outside of the active site. Thr194 is rotated with respect to the equivalent Thr199 of the castor desaturase and shows two distinct side chain conformations, the weaker of which points toward the diiron site and enables the interaction with Glu224. The role of Thr194 in desaturase catalysis is unclear, but mutation of Thr199 in the castor enzyme to an aspartic acid resulted in a switch from desaturase to oxidase chemistry (40). A conserved threonine at this position is also seen in MMO, and the side chain has been reported in alternative rotameric conformations in crystal structures (41, 42). In MMO, as with the desaturase, the exact role of this residue is unclear, but it has been suggested to play a role in proton transfer (41). The conformational change in Thr194 between the oxidized and reduced desaturase structures and its potential interaction with the oxidized active site suggest that the residue could also play a role in the catalytic cycle of the desaturases.

The active site changes that occur on oxidation of the castor desaturase have previously been analyzed by spectroscopic methods. Electron nuclear double resonance (ENDOR) studies (43) of the cryoreduced desaturase and desaturase-substrate complex indicated that the major conformation of the oxidized active site has a histidine and a water molecule bound to each iron ion. In the crystal structure only Fe2 binds water, and only Fe1 interacts directly with a histidine residue. The difference in water coordination might be explained by the presence of the bound fatty acid ligand, which would be expected to replace a water molecule as stated above. Although the crystal structure does not show the predicted histidine coordination, it is interesting to note that 14N ENDOR spectra for the desaturase and desaturase-substrate complexes are much less resolved and intense than those of the structurally similar cryoreduced diiron cluster in ribonucleotide reductase R2 subunit. This fact was interpreted to reflect differences in the His-iron bonds in the oxidized desaturase compared with other diiron proteins.

**Comparison of the oxidized diiron centers of the desaturase, MMO, and rubrerythrin (Fig. 7)** shows, however, that there are several features of the desaturase diiron site that are unique. The first and perhaps most significant of these is the carboxylate shift. The total loss of iron coordination by Glu224 results in a significantly different symmetry of the oxidized desaturase compared with that in the other oxidized enzymes, with each iron retaining one bidentate glutamic acid and one bridging. The loss of histidine coordination to Fe2 is also unique to the desaturase; in MMO and ribonucleotide reductase the histidine coordination remains unchanged upon oxidation, whereas in rubrerythrin it is the histidine coordinating Fe1 that is disengaged. Water coordination is different in each of the three proteins. MMO, in the presence of the bound acetate ligand, has only a single water in the active site, coordinating Fe1, whereas in the absence of acetate an additional weakly bound water
bridges the two irons. The oxidized ruberythrin structure has no water molecules coordinating to the iron center.

The Substrate-binding Sites—Despite the highly similar overall structures of the desaturases from castor and ivy, the product outcome from the two enzymes differ markedly; castor desaturase gives almost exclusively 18:1Δ⁹ (and 16:1Δ⁹ when presented with 16:0 substrate), whereas the ivy enzyme is multifunctional but predominantly results in 16:1Δ⁴. The ability to manipulate the substrate specificity of desaturases is useful for biotechnological applications, and mutagenesis experiments have been performed based on the castor desaturase structure in conjunction with multiple sequence alignments (19, 20, 45). Some success was achieved, particularly with respect to chain length specificity, but many questions, such as how the position of double-bond insertion is determined, remain to be resolved. The ivy desaturase structure provides a unique opportunity for substrate-binding site comparison of two desaturases with differing specificities.

The two structures have been examined in detail for differences that could potentially contribute toward substrate selectivity and product outcome, and the most significant were found to be clustered around two regions: the presumed ACP-binding interface (33) and the bottom of the substrate-binding cavity. The residues that differ between the two enzymes and could potentially interact with either ACP or the acyl group are shown in Fig. 8. Those lining the substrate-binding cavity (Fig. 8A) correspond mainly to residues that have been studied by mutagenesis, and the structure is consistent with published data. Arg¹¹², Cys¹¹³, and Ile¹⁷⁴ correspond to a threonine (Thr¹¹⁷), leucine (Leu¹¹⁸), and proline (Pro¹⁷⁹), respectively, in the castor enzyme. The T117R, L118C, and P179I mutations have previously been introduced into the castor desaturase and were all shown to cause increased specific activity for 16:0-ACP relative to 18:0-ACP while retaining the Δ⁹ regioselectivity (19, 20, 45). Each of these residues lie close to the predicted position of the methyl end of the fatty acid substrate and act as determinants of the chain length that can be accommodated. The fourth difference noted, Ser¹⁷⁶, which corresponds to Thr¹⁸¹ in castor, is a more conservative substitution and hence less likely to effect a specificity change.

It is interesting to note that, although the ivy desaturase exhibits a preference for the shorter 16:0 substrate, its ability to insert a double bond at the Δ⁴ position actually requires it to accommodate more of the fatty acid, i.e. 12 carbons in the cavity beyond the diiron site. In this context it is therefore surprising that the ivy desaturase contains bulkier residues, which should effectively shorten the cavity, relative to the castor desaturase. Comparison of the two structures suggests a possible solution to this problem; in the castor enzyme the Oy of residue Thr¹¹⁷ can partly block the ability of the carbon chain to move lower down, and although the corresponding Arg¹¹² in the ivy enzyme is larger, the side chain is actually pointing away from the substrate, and the obstruction from the Thr¹¹⁷ oxygen is removed. This residue exchange, in combination with the leucine to cysteine substitution at residue 113 (Leu¹¹⁸ in castor), produces additional space at the base of the cavity in the ivy desaturase, and we propose that this allows accommodation of additional methylene groups at the bottom of the cavity.

Analysis of residues that could potentially interact with the acyl carrier protein or the carboxyl end of the fatty acid is more difficult because of the larger area involved in the desaturase, but there are several differences between these regions of the two structures that merit mention. The predicted binding site of ACP (33) lies along a flexible loop and helix at the surface of the desaturase dimer. On the section of the helix that is most likely to interact with the substrate or ACP are shown as ball-and-stick representations.

![FIGURE 8. The superimposed ivy (blue) and castor (green) desaturases, with the fatty acid substrate modelled in yellow. Shown are (A) the base of the substrate-binding cavity, and (B) the region surrounding the predicted ACP binding site. Residues that differ between the two structures and likely to interact with the substrate or ACP are shown as ball-and-stick representations.](image-url)
Lys	extsuperscript{328}, correspond to Asp	extsuperscript{225}, Arg	extsuperscript{229}, and Arg	extsuperscript{333} in the castor enzyme (Fig. 8B). There is also a cluster of potentially interesting substitutions found at the binding site of the carboxyl end of the modeled substrate and the pantetheine group of ACP. In particular, residues Lys	extsuperscript{375} (Asp	extsuperscript{280} in castor) and Thr	extsuperscript{378} (Ser	extsuperscript{283} in castor) are pointing into the mouth of the substrate-binding cavity and are both very likely to interact with either the thioester or pantetheine group. We hypothesize that residues in this region contribute to regioselectivity of the enzyme, because a different position of the substrate carboxyl group is required to align the fatty acid for Δ4 or Δ9 desaturation relative to the oxidizing species on the diiron center, the location of which is determined by the four helix architecture of the enzyme. Experiments are currently underway to test these hypotheses regarding the roles of these residues in determining regioselectivity and chain length specificity of the desaturases.

Acknowledgment—We thank Dr. P. Buist (Carleton University, Ottawa, Canada) for helpful discussion.

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