Di-iron clusters within the active sites of enzymes facilitate the binding of molecular oxygen and its derivatives and are able to perform redox chemistry, which results in a range of chemical outcomes (Edmondson and Juynh, 1996). All di-iron enzymes characterized to date belong to one of two separate classes, one soluble and the other membrane-bound (Shanklin and Somerville, 1991). Both classes have the ability to catalyze the oxidation of unactivated C–H bonds to give a range of chemical outcomes (Shanklin and Cahoon, 1998; Fox et al., 2004). For instance, both soluble and membrane di-iron enzyme classes contain desaturase enzymes that perform the stereo- and regioselective introduction of Z-(cis) double bonds into unactivated lipid acyl chains. The reactions are thought to proceed via a radical mechanism initiated by abstraction of a specific hydrogen from substrate (Buist, 2004). Double bond formation ensues via the abstraction of a second neighboring hydrogen. As predicted by Bloch (1969) and subsequently confirmed by x-ray crystallography (Lindqvist et al., 1996; Bai et al., 2015), the boomerang shape of the substrate binding channel within the desaturase drives the formation of the (Z)-olefinic fatty acids.

There is a diverse constellation of chemical outcomes performed by variant enzymes that are structurally related to the prototypical desaturase. The membrane-bound di-iron–containing plant fatty acid desaturase (FAD) family of FAD2 variant enzymes perform a variety of chemical transformations. Using oleate as substrate, either desaturated or hydroxylated products are obtained; using linoleate as a substrate, the corresponding epoxide, a conjugated double bond, or an acetylenic bond can be produced. Changes in chemoselectivity are based on a relatively small number of amino acid sequence differences that presumably alter the relative orientation of the substrate with respect to the active site oxidant (Bhar et al., 2012). For instance, changes to only four amino acid side chains was sufficient to predominantly convert a FAD2 into a hydroxylase and vice versa (Broun et al., 1998; Broadwater et al., 2002). Despite our increasing understanding of specificity determining residues within the FAD2-related di-iron
enzymes, further interpretation has been hindered by the lack of structural information for these enzymes. Recently published structures of several mammalian membrane-bound desaturases suggest it will be possible to solve one of the plant FAD2 class at some point and we will be able to correlate changes to the enzyme structure with distinct functional outcomes (Bai et al., 2015; Wang et al., 2015).

The soluble class of desaturase enzymes exemplified by the castor (Ricinus communis) Δ⁹18:0-ACP desaturase (Lindqvist, 2001) has been shown to contain members that display a variety of chain-length specificities and regioselectivities (Shanklin et al., 2009). Mechanisms have been proposed for both chain length specificity (Cahoon et al., 1997; Whittle and Shanklin, 2001) and for regioselectivity (Guy et al., 2011). During our studies on regioselectivity, we engineered a triple mutant of the castor acyl-ACP desaturase (T117R/G188L/D280K) that converts stearoyl-ACP into an allylic alcohol transisomer (E)-10-18:1-9-OH via a (Z)-9-18:1 intermediate (Whittle et al., 2008). This work described a soluble desaturase acting as an olefin oxygenase similar in behavior to that displayed by another soluble di-iron protein, methane monooxygenase (Gherman et al., 2004). We showed that the conversion of (Z)-9-18:1 substrate to (E)-10-18:1-9-OH product by castor desaturase T117R/G188L/D280K proceeds via hydrogen abstraction at C-11 and highly regioselective hydroxylation (>97%) at C-9 (Whittle et al., 2008). The ¹⁸O-labeling studies show that the hydroxyl oxygen in the reaction product is exclusively derived from molecular oxygen.

This work was initially designed to evaluate the individual contributions of T117R, G188L, and D280K in castor desaturase to allylic alcohol formation. During these experiments, we discovered a novel dioxygenase reactivity of the soluble desaturase that results in the conversion of oleoyl-ACP to erythro-9,10-dihydroxystearate. The same product was found in TMS-derivatized methyl esters from castor seed where it constitutes ~0.7% of the total fatty acids.

RESULTS

As part of our continuing structure–function analysis of di-iron enzymes, we analyzed the contributions of
each of the mutations within the castor desaturase T117R/G188L/D280K triple mutant that converts oleoyl-ACP into (E)-10:18:1-9-OH (Whittle et al., 2008). Each of the individual mutants was constructed and tested for its activity using oleoyl-ACP as substrate. In each case, the product profiles were determined by gas chromatography–mass spectrometry (GC–MS) analysis. The results are shown in Figure 1. The GC elution profile of substrate is shown in Figure 1A and features a peak corresponding to 18:1 D9 methyl ester (peak 1). A minor shoulder peak can be attributed to 18:1 D11 (peak 2) and is a well-known artifact of the expression system. As shown in Figure 1B, the triple mutant T117R/G188L/D280K converted most of the oleoyl-ACP substrate into a mixture of the Z(cis)18:1 D10 9OH (peak 3) and E(trans)18:1 D10 9OH allylic alcohol (peak 4) isomers, with the E form predominating by ~3-fold over the Z form.

The Novel Fatty Acid Product (Peak 5) Is 9,10-Dihydroxystearate

Mass-spectral analysis of the peak-5 product produced by the T117R mutant (Fig. 1C) revealed a molecular ion of 474 AMU, consistent with an 18C fatty acid methyl ester containing two silylated hydroxyl groups (Fig. 2A). Fragmentation of the product between the two silyl groups produced fragments of 259 AMU for the carboxyl-containing fragment and 215 AMU for the methyl-containing fragment (diagrammed in Fig. 2B), consistent with the presence of vicinal hydroxyl groups at C9 and C10. The identity of the peak-5 product was confirmed by comparison of its fragmentation pattern with that of a silylated authentic commercial standard of erythro-methyl 9,10-dihydroxy stearate (Fig. 2C). Analysis of the peak-5 product from the D280K mutant also showed the same fragmentation pattern.

9,10-Dihydroxystearate Produced by the T117R Mutant Is Solely in the erythro Configuration

Fatty acids containing vicinal midchain hydroxy groups may exist as threo or erythro diastereoisomers (Fig. 3). To distinguish between these possibilities, we compared the GC elution times of the novel product from T117R with those of authentic threo- and erythro-9,10-dihydroxystearate standards (Fig. 4, A–C, respectively). The T117R product eluted as a single defined peak without any detectable shoulders (Fig. 3A) and coeluted with authentic erythro standard (Fig. 4C). The authentic threo standard (Fig. 4B) eluted ahead of that of the T117R product (Fig. 4A). When a small amount of the T117R product was mixed with either the threo standard (Fig. 4D) or the erythro standard (Fig. 4E), two peaks were seen for the sample spiked with threo standard whereas a single coeluting peak was seen for the sample spiked with erythro standard. These results
confirms the assignment of the T117R product as \textit{erythro}-9,10-dihydroxystearate.

The Hydroxyl Oxygens at Both C9 and C10 Are Derived from Molecular Oxygen

The oxygen atoms in either of the two hydroxyl groups could in principle arise from water or molecular oxygen (Fig. 5). To distinguish between these possibilities, T117R, oleoyl-ACP, and all assay components were first degassed by multiple gas exchange cycles employing vacuum and O2-free argon with the use of a Schlenk line (Arnold and Bohle, 1996) to remove residual atmospheric \textsuperscript{16}O\textsubscript{2} from the sealed reaction vials. Assay reactions were subsequently incubated in the presence of \textsuperscript{16}O\textsubscript{2} or \textsuperscript{18}O\textsubscript{2}. We used mass-labeled \textit{18:1} \textit{d}_{11,11} oleoyl-ACP for these assays to ensure the product we observed was derived from the enzymatic reaction rather than from endogenous oleate contaminant. Analysis of the methylated silylated products from reaction under air yielded the expected 217 and 259 AMU products (the methyl fragment increased by 2 AMU relative to unlabeled product results from the substitution for the two hydrogens at C11 for deuterons; Fig. 6). The same experiment performed under \textsuperscript{18}O\textsubscript{2} resulted in the production of fragments of 219 and 261 AMU, consistent with the incorporation of one \textsuperscript{18}O at each of the hydroxyl positions.

The Formation of 9,10-Dihydroxystearate from Oleate Is the Result of a Dioxygenase Reaction

The incorporation of molecular oxygen at the 9 and 10 positions of oleate could in principle result from a single dioxygenase reaction, or from two sequential monooxygenase reactions. To distinguish between these possibilities, we degassed the samples as described above and performed a reaction under an atmosphere containing an equimolar fraction of \textsuperscript{16}O\textsubscript{2} and \textsuperscript{18}O\textsubscript{2} (Fig. 7B) and performed MS on methylated acetone derivatives of the product (Fig. 7E). Acetone derivatives were used because they protect vicinal hydroxy groups while maximizing the detectable mass ion of the product. If the reaction operates via a dioxygenase mechanism, then the oxygen atoms at both hydroxyl positions should derive exclusively from either \textsuperscript{16}O\textsubscript{2} or \textsuperscript{18}O\textsubscript{2}, resulting in either M or M+4 species. Alternatively, if the mechanism employs two sequential monooxygenase reactions, a 1:2:1 pattern of M:M+2:M+4 would be expected by random incorporation of either \textsuperscript{16}O or \textsuperscript{18}O at each hydroxyl position. Consistent with a dioxygenase mechanism, reactions performed under an equimolar mix of \textsuperscript{16}O\textsubscript{2} and \textsuperscript{18}O\textsubscript{2} yielded only M and M+4 peaks (355 and 359), with no detectable 357 species (Fig. 7B). Individual control \textsuperscript{16}O\textsubscript{2} and \textsuperscript{18}O\textsubscript{2} reactions only showed the expected 355 and 359 major species accompanied by minor peaks at M+1 and M+2 that approximate the natural abundance of \textsuperscript{13}C (Fig. 7, A and C, respectively). That M+1 and M+2 peaks originate from natural \textsuperscript{13}C was confirmed by the fragmentation of equivalent derivatives of an authentic

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**Figure 4.** The 9,10-dihydroxystearate produced by the castor T117R mutant is solely in the \textit{erythro} configuration. GC images of 9,10-dihydroxy-stearates are compared for the reaction product of T117R (A) to those of standards: \textit{threo} configuration (B), the \textit{erythro} configuration (C), a mixture of the T117R product and the \textit{threo} standard (D), and the T117R product and the \textit{erythro} standard (E).
erythro-9,10-dihydroxystearate, which showed the same proportions of M, M+1, and M+2 species (Fig. 7D).

The Native Castor Desaturase Can Convert Oleoyl-ACP to 9,10-Dihydroxystearate

The formation of dihydroxystearate with selected mutated desaturases prompted us to probe for the formation of this compound by the wild-type enzyme. Interestingly, using a prolonged time of incubation (240 min) with oleoyl-ACP as substrate, we were able to identify production of 9,10-dihydroxystearate (peak 5) at low levels (Fig. 8). This compound was accompanied by lesser amounts of E 18:1 10 9 OH (peak 4).

Castor Oil Contains Erythro-9,10-Dihydroxystearate

The observation that the native castor desaturase can produce small amounts of 9,10-dihydroxystearate correlates well with an early report by King (1942), who isolated a small amount of 9,10-dihydroxystearate from castor oil. We sought to confirm this observation and analyzed a fatty acid extract of castor seeds by GC–MS after methylation and silylation. Chromatograms of castor seed fatty acid derivatives (Fig. 9A) showed the expected common C16 and C18 fatty acids, along with a major peak of ricinoleic acid that is followed by a small discrete peak (labeled “8” in Fig. 9A, inset) of ∼0.7% (of total fatty acids), which corresponds to the elution time of disilylated methyl 9,10-dihydroxystearate. Mass spectral analysis of this peak revealed fragments of 215 and 259 AMU confirming its assignment as 9,10-dihydroxystearate (compare Fig. 9B with Fig. 2, A and C).

Based on the in vitro assays using purified enzyme reported above, we hypothesize that this 9,10 dihydroxystearate arises from the dioxygenation of oleoyl-ACP product of the stearoyl-ACP desaturase. If this were the case, the 9,10-dihydroxystearate would be in...
the erythro form as originally proposed (Morris and Crouchman, 1972). We therefore conducted coelution studies with authentic threo or erythro standards (Fig. 9, C–E). The 9,10 dihydroxystearate isolated from castor eluted as a single peak (Fig. 9C) with the same mobility as that of the authentic erythro standard (Fig. 9E). By contrast, two peaks were seen in the spiking experiment using the threo standard (Fig. 9D).

DISCUSSION

Stereo-selective dihydroxylation reactions are important to the chemical industry (Borrell and Costas, 2017) because diols serve as valuable synthons. The osmium-based asymmetric dihydroxylation reaction (Crispino and Sharpless, 1993) is a prominent example of controlled olefin oxidation and was (in part) recognized by the award of the 2001 Nobel Prize in Chemistry to its inventor, Karl B. Sharpless. In addition, biocatalytic diol formation from aromatics by whole-cell mutant Pseudomonas cultures has furnished the synthetic chemist with a variety of enantiomerically pure cyclohexadiene-cis-diols. (Hudlicky and Thorpe, 1996). Much effort has also been expended to develop iron-based biomimetic catalytic methodology for this reaction (Oloo and Que, 2015). Herein, we report the details of our investigation into a “green chemical approach”: the castor Δ^9:18:1-ACP desaturase-mediated syn-dihydroxylation of an unactivated alkene in the form of oleoyl-ACP to erythro-9,10-dihydroxystearoyl-ACP.

Stearoyl-ACP desaturase belongs to the nonheme di-iron subclass of oxidative enzymes that have been shown to mediate a variety of chemical transformations including dehydrogenation and mono-oxygenation. Typical products include primary, secondary, and allylic alcohols in addition to the conversion of double bonds to epoxides (Wallar and Lipscomb, 1996). However, a di-iron center performing dioxygen chemistry to convert a double bond to a vicinal diol as reported here is without precedent. The closest comparable example we are aware of is arylamine oxygenase (CmlI) from the chloramphenicol biosynthesis pathway, which incorporates two oxygens from O_2 into the aryl-nitro product; however, this occurs in two consecutive mono-oxygenations.

Figure 7. The 9,10-dihydroxy stearate formation is the result of a single dioxygenase reaction. GC images and corresponding mass spectra of acetonide derivatives of 9,10 dihydroxy stearate from reactions carried out under 16O_2 (A), equimolar 16O_2 and 18O_2 (B), and 18O_2 (C). Also depicted is an authentic erythro 9,10 dihydroxy stearate standard (D) along with a diagram of its fragmentation (E).

Figure 8. Upon prolonged incubation, the castor wild-type desaturase can convert 18:1 substrate to erythro-9,10-dihydroxystearate. Peak identities: Z18:1Δ9 (1), Z18:1Δ11 (2), E18:1Δ10 9OH (4), and 9,10-dihydroxystearoyl-ACP (5).
We envision the conversion of alkene to vicinal erythro-diol in this work to be mechanistically related (Fig. 4) to that described for Rieske cis-diol-forming dioxygenases (Ensley et al., 1982; Karlsson et al., 2003). More specifically, we envision involvement of a bridged hydroperoxo-di-iron species similar to that proposed by Solomon and Smec (Chalupský et al., 2014) for the conversion of stearate to oleate by two consecutive hydrogen atom abstractions: “-CH2-CH2-” to “-CH=CH-.” When presented with an alkene moiety, the vinyl hydrogens are unavailable for abstraction for steric reasons and this same species is forced to transfer two oxygen atoms to substrate as shown in Fig. 4 (Pathway 1). Our oxygen-labelling experiments rule out an epoxidation/hydrolysis route (Pathway 2). It is possible that our T117R mutant may change the molecular architecture of the substrate binding cavity, altering the relative orientation of the substrate with respect to the hydroperoxo-di-iron group and facilitating deoxygenation relative to the wild-type enzyme. That the diol is produced as the erythro-diastereoisomer, in which both hydroxy groups occur on one face (Fig. 3), is consistent with the geometry of the active site substrate-binding cavity with respect to the di-iron active site oxidant (Lindqvist et al., 1996), in which steareate binds in a quasi-eclipsed conformation at C9 and C10, projecting the pro-(R) hydrogens toward the active site oxidant (Behrouzian et al., 2002). Future availability of a crystal structure of the T117R mutant in complex with bound oleoyl-ACP, or of the T117R mutant alone or with substrate bound as previously modeled (Whittle et al., 2008), would be useful starting points for probing mechanistic models using computational methods such as density functional theory. Indeed, homology modeling was recently shown to be a useful approach for elucidating selectivity mechanisms of desaturase enzymes such as FAD2 and FAD3 (Cai et al., 2018).

The low levels of 9,10-dihydroxystearate in castor suggests that this system is not optimized to produce this particular product. Higher levels of the diol may accumulate via enzymes with active site geometries that permit more efficient dioxygenation. *Cardamine impatien*s is an example of a plant that accumulates ~25% of 9,10-dihydroxystearate (and its chain-elongation products) in its seed oil (Mikolajczak et al., 1964). It is tempting to speculate that it contains a desaturase that has undergone mutation/selection to optimize the production of the diol from the initial alkene product. Examples of desaturases with multiple sequential oxidation activity include English ivy (*Hedera helix*) that performs Δ9-followed by Δ4 desaturation on stearyl-ACP (Guy et al., 2007); FM1, a fungal membrane desaturases that sequentially inserts a Δ12 followed by a Δ15 double bond into oleoyl-phosphatidyl ethanolamine (Cai et al., 2018); and an insect multifunctional enzyme that functions as a Δ11 desaturase, Δ11 acetylenase, and Δ13 desaturase (Serra et al., 2007).

Major oxygenated fatty acids such as ricinoleic- and vernolic acids are typically produced in the endoplasmic reticulum by variant FAD2 membrane-bound desaturases (van de Loo et al., 1995; Lee et al., 1998). On the other hand, fatty acids with unusual double bond positions such as 16:1Δ4, 16:1Δ9, and 18:1Δ6 are synthesized within the plastid (Shanklin and Cahoon, 1998). Thus, the production of oxygenated fatty acids such as the erythro-9,10-dihydroxystearate in the plastid as reported here is very unusual if not unique. It is likely that in species with high levels of accumulation such as...
C. impatiens, there exists a variant acyl-ACP thioesterase that cleaves the vicinal diol fatty acid from its ACP adduct in addition to specialized acyltransferases and other components that facilitate its transfer from the plastid to triglyceride storage lipids. More than 70 years ago, 9,10-dihydroxystearate was reported as a component of castor oil (King, 1942) at ~1% of the total fatty acids (Sreenivasan et al., 1956). The stereochemistry of the diol was later determined to be the erythro configuration (Morris and Crouchman, 1972). Consistent with these earlier reports, castor oil samples evaluated in this work contained ~0.7% of erythro-9,10-dihydroxystearate. That the wild-type castor desaturase can produce this compound was an entirely unanticipated result and resolves a long-standing mystery. In addition, our results underscore the remarkable plasticity of the nonheme di-iron catalytic center found in the desaturase family of enzymes. It appears that subtle changes in the active site architecture found in these versatile oxidants can allow new reaction pathways to emerge. Further detailed mechanistic work is needed to understand the relationship between reaction outcome and details of the active site architecture.

MATERIALS AND METHODS

Mutant Construction

Synthesis of the castor (Ricinus communis) desaturase triple mutant T117R/G168L/D280K and D280K single mutants were as described in Whittle et al. (2008) and Guy et al. (2011). The single mutants T117R and G168L were identified by mutagenesis-selection experiments (Whittle and Shanklin, 2001). The open reading frames were introduced into pET9d using XbaI and EcoRI restriction sites and the resulting clones were validated by sequencing.

Mutant Analysis

Desaturases, and variants thereof, were overexpressed in Escherichia coli BL21(DE3) with the use of pET9d. Recombinant desaturase was enriched to >90%-purity by 20CM cation exchange chromatography (Applied Biosystems). Desaturation reactions (600 μL; Cañon and Shanklin, 2000) were performed by incubation of the desaturase with 18:0- and 18:1-ACP substrates in the presence of recombinant spinach ACP-I (Beremand et al., 1987). Uniformly deuterated stearate was obtained from Cambridge Isotope Laboratories and 9,10 d2 oleate and 11,11 d2 oleate was obtained from the collection of Tulloch (1983). Experiments reported herein were replicated three or more times and representative results are presented.

Fatty Acid Analysis

Fatty acid methyl esters (FAMEs) were prepared by addition of 2 mL of 1% (v/v) NaOCH3 in methanol and incubated for 60 min at 50°C. Fatty acid methyl esters were extracted twice into 2 mL of hexane after addition with 100 μL of glacial acetic acid. Hexane was evaporated to dryness under a stream of N2, and samples were resuspended in hexane for GC analysis. FAMEs were dried and resuspended in 100 μL of N,N′-bis(trimethylsilyl) trifluoroacetamide (BSTFA) and trimethylchlorosilane (TMCS; Supelco) for 45 min at 60°C to create trimethyl silyl derivatives. Samples were analyzed with an HP5890 gas chromatograph (Agilent) fitted with a 60 m × 250-μm SP-2340 capillary column (Supelco). The oven temperature was raised from 100°C to 160°C at a rate of 25°C min⁻¹ and from 160°C to 240°C at a rate of 10°C min⁻¹ with a flow rate of 1.1 mL min⁻¹. Mass spectra were analyzed using an HP5973 mass selective detector (Agilent). For 18O experiments, oxygen was removed from the sample cell by repeated evacuation and purging of the cell with O2-free argon using a Schlenk line. Two mixtures were prepared—one containing desaturase enzyme, buffer, ferredoxin NADP⁺ reductase, and substrate, and the other containing ferredoxin and NADPH. The two anaerobic mixtures were transferred to sealed reaction vials containing an atmosphere composed of either 16O₂, 18O₂ (Cambridge Isotope Laboratories), or an equimolar mixture of 16O₂ and 18O₂. Reactions were terminated by the addition of toluene, and fatty acids were esterified and silylated as described above for experiments designed to fragment the fatty acid to reveal the position of the vicinal hydroxyl groups. Alternatively, for the labeled oxygen experiments designed to determine the reaction mechanism, fatty acids were converted to methyl esters after which vicinal hydroxy groups were converted to their acetonide derivatives (Singh et al., 2008). To achieve this, methyl ester samples were dried under nitrogen and resuspended in 40 μL of 4 μL of ZrCl4 catalyst in diethyl ether, 200 μL of dichloromethane (CH2Cl2), and 5 μL of dimethoxypropane. The mixture was incubated with shaking at 22°C for 2 h. The mixture was extracted with 3 mL of chloroform (CHCl₃) and 1 mL of water, separated by centrifugation (at 1,500 g for 5 min) and the lower phase was collected and dried under nitrogen before resuspension in hexane for GC–MS analysis. Samples were analyzed on an HP6890/5973 GC–MS equipped with a 30 m × 250-μm HP-SP-2340 capillary column (Supelco). Oven temperature was held at 100°C for 2 min, raised to 30°C at the rate of 20°C min⁻¹, and held for 2 min.

Accession Numbers

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession number M59857.

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