ABSTRACT

Second Sphere Effects of O_2 Activation in Stearoyl-Acyl Carrier Protein Δ9 Desaturase

Stearoyl-acyl carrier protein Δ^9 desaturase (Δ9D) from *Ricinus communis* converts stearic acid into monounsaturated oleic acid and functions to maintain the lipid composition of the cell membrane. While the desaturase active site glutamate and histidine residues are crucial to catalysis, there are a number of conserved "second sphere" residues. One such residue is Threonine 199 found ~5Å from the diiron center and it has been hypothesized to stabilize the peroxo intermediate formed during catalysis. In this work, the role of this residue has been probed through studies of a series of mutants. These mutations show a lower k_{cat} and an increase in the rate of decay of the peroxo intermediate, providing evidence of stabilization of the intermediate.

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COVER SHEET

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College of Agricultural & Life Sciences
Senior Honors Thesis Final Report
Honors in Research

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Second Sphere Effects of O_2 Activation in Stearoyl-Acyl Carrier Protein Δ9 Desaturase

Summary

Stearoyl-acyl carrier protein Δ^9 desaturase (Δ9D) from *Ricinus communis* converts stearic acid into monounsaturated oleic acid and functions to maintain the lipid composition of the cell membrane. While the desaturase active site glutamate and histidine residues are crucial to catalysis, there are a number of conserved "second sphere" residues. One such residue is Threonine 199 found ~5Å from the diiron center and it has been hypothesized to stabilize the peroxo intermediate formed during catalysis. In this work, the role of this residue has been probed through studies of a series of mutants. These mutations show a lower k_{cat} and an increase in the rate of decay of the peroxo intermediate, providing evidence of stabilization of the intermediate.

Introduction

In plants, fatty acid biosynthesis occurs in the chloroplasts of green tissue or in the plastids of non-photosynthetic tissues. The primary products are acyl carrier protein (ACP) esters of saturated palmitic and stearic acids. Fatty acid desaturases are iron-containing multiprotein complexes that insert double bonds into fatty acyl chains after their biosynthesis[6-8]. They provide lipid precursors to cell membranes, to nutritionally essential polyunsaturated fatty acids and to prostaglandins[9]. Abnormal lipid metabolism can lead to diseases such as hypertension, non-insulin-dependent diabetes, obesity, neurological pathologies and many others[1]. There is also increased interest in fatty acid biosynthesis in plants because of the possible use of plant oils as renewable sources for reduced carbon[10].

Stearoyl-acyl carrier protein Δ^9 desaturase (Δ9D) from *Ricunis communis*, the castor seed, belongs to the family of diiron enzymes that catalyze a variety of oxygen-dependent reactions. This soluble desaturase is responsible for the biosynthesis of oleic acid, the most abundant unsaturated fatty acid. It introduces a cis double bond between carbon 9 and 10 of stearoyl-ACP, converting it into oleoyl-ACP[13]. This reaction is highly regiospecific and stereospecific. Four proteins are required for desaturase activity: acyl carrier protein (ACP), ferredoxin, ferredoxin reductase and stearoyl-ACP Δ^9 desaturase (Δ9D). The substrates for Δ9D are acyl-acyl carrier proteins (acyl-ACP). This is also an oxidase reaction where 4 electrons are used to convert O_2 into 2 moles of water[1]: $O_2 + 4e^- + H_2O \rightarrow 2H_2O$ (See Fig. 1).

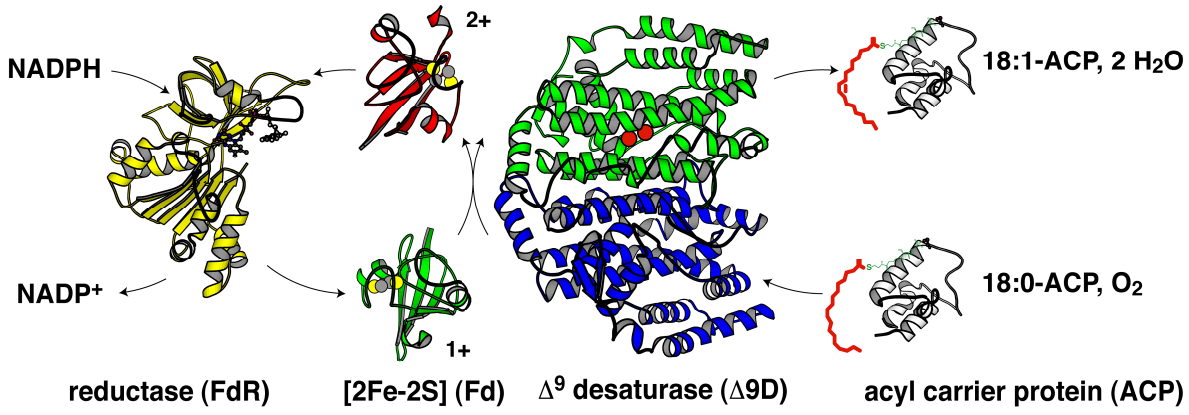


Fig 1. Δ9 – 18:0-ACP desaturase electron transport chain.

The Δ9D enzyme is a homodimer consisting of two 42kDa subunits[3] (Fig.2). The active site is a diiron center ligated by histidine and glutamate and residues within a four helical bundle that has high structural similarity to ribonucleotide reductase[5] and multicomponent monooxygenases from the same family of diiron enzymes. The four helical bundle is needed for binding and activating oxygen, which is a reaction this class of diiron enzymes have in common. It is also universally conserved and responsible for binding the two iron atoms (See Fig. 1). The diiron center is in a bent channel in the enzyme at a depth where carbon 9 and 10 of the 18 carbon fatty acid will be situated during catalysis. Reducing equivalents from NADPH are transferred from ferredoxin reductase to ferredoxin and then to Δ9D[13]. In the presence of O_2, hydrogen at C-9 and C-10 are removed from stearoyl-ACP (18:0-ACP, 18:0 designates an 18 carbon fatty acid with no double bond) to give oleoyl-ACP (cis-Δ^9-18:1-ACP, cis-Δ^9-18:1-ACP is an 18 carbon fatty acid with a cis double bond at the C-9 position) with complete fidelity of double bond position and the correct cis stereochemistry. While the active site glutamate and histidine residues are crucial to catalysis, there are a number of conserved "second sphere" residues. Some of these residues are hydrogen bonded to ligating residues and are

thought to play a role in electron transfer[2]. One such residue is Threonine 199 found ~5Å from the diiron center[3] (Fig. 3) and has been hypothesized to stabilize the peroxo intermediate formed during catalysis.

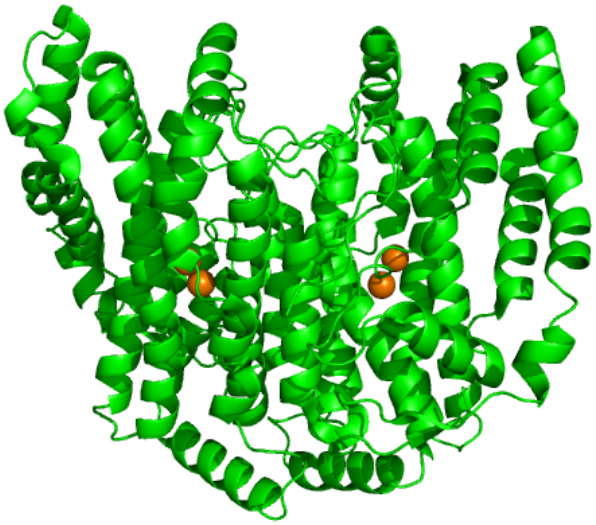


Fig. 2 Δ9D Homodimer

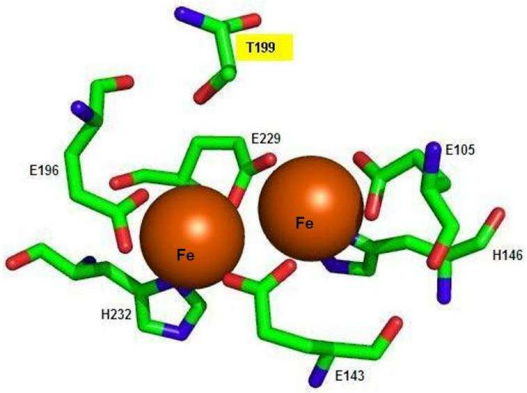


Fig. 3 Active Site of Δ9D. Threonine 199 is highlighted and is ~5Å from the diiron center.

While Δ9D is also the most studied desaturase, many details of the desaturase catalysis, such as contributions of protein interactions and reactivity of the required diiron centers, are not well understood. Work on desaturases is important as it suggests a source of renewable carbon sources. With deeper understanding of the mechanistic details, protein engineering can be used to modify enzymes into generating fatty acids of desired length and degree of saturation, which is useful for industrial and commercial purposes. Understanding the reaction mechanism would be useful to human medicine and health-related studies as the research can also provide insights into abnormal lipid metabolism which causes diseases such as obesity, hypertension and diabetes.

Previous work done in the Fox lab has identified an oxygen activated peroxo intermediate when Δ9D is reduced with dithionite instead of ferredoxin, the biological redox partner. This μ-1,2-peroxo-bridged diferric complex is designated peroxoΔ9D. The peroxo intermediate formed in the wild type Δ9D is quite stable with a half life ($t_{1/2}$) for decay of ~26 min and its decay does not result in product. This peroxo intermediate and the intermediate that is formed during catalysis are likely stabilized by the interactions with the surrounding amino acid residues. The mechanism of catalysis is still unclear. Threonine 199 has been hypothesized to stabilize the peroxo intermediate formed during catalysis and mutations made to this position are expected to result in peroxo intermediates that are not as stable.

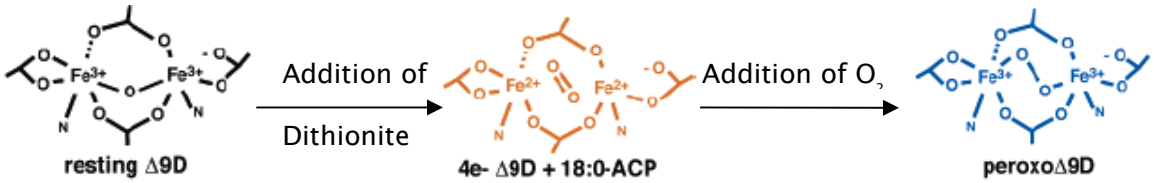


Fig 4. Formation of peroxoΔ9D intermediate after addition of dithionite

Recent work by Guy *et al*. has begun to probe the function of T199[6]. T199 was changed to aspartic acid (T199D) and glutamic acid (T199E). These mutations resulted in a thousand fold decrease in the rate of desaturation with a ~30 fold increase in catalase activity. The increase in catalase activity was hypothesized to occur since the active site of the desaturase has been shown to be structurally similar to that of rubrerythrin, which is an enzyme with catalase activity. Threonine is a poor proton donor. In Δ9D, it is situated ~4.5Å from the diiron site, while at the same residue position in rubrerythrin, it is glutamic acid which facilitates proton transfer rather than threonine. While this work has

focused on the effect of this residue on catalase chemistry, no work was focused on the effects of mutation on the catalytically relevant pathway, oxygen activation and subsequent desaturation. Mutagenesis of this and other surrounding residues may provide insight into the mechanism of this important class of enzymes and provide a more complete understanding of the mechanistic details of desaturase reaction.

Experimental Procedures

1. Site Directed Mutagenesis

The sequence of the Δ9D gene is known. The gene for Δ9D has been cloned into pET-3d, with the resulting plasmid known as pRCMD9. The desired mutants were created using site directed mutagenesis[3]. Primers were designed where the threonine residue was replaced with alanine (T199A), serine (T199S), asparagine (T199N), glutamine (T199Q), aspartic acid (T199D), glutamic acid (T199E). The mutant gene was then sequenced to ensure the correct mutation has been obtained.

2. Protein Purification

The recombinant plasmid was used to transform *Escherichia coli* strain BL21(DE3). The protein was overexpressed by induction with IPTG. The cells were then broken by sonication and the cell free extract fractionated by DEAE anion exchange. Fractions containing Δ9D were identified using denaturing polyacrylamide gel electrophoresis and concentrated using ultrafiltration on a YM30 membrane (Amicon). The concentrated protein was then applied to a Sephacryl S-100 column and fractions containing Δ9D were identified and concentrated using ultrafiltration.

3. Characterization of Mutants.

Steady state catalysis assays

Steady state catalysis assays were done on the mutants to study the K_m and k_{cat} parameters. In a typical assay, 1μM of Δ9D, 1μM of ferredoxin, 0.169μM of ferredoxin reductase, varying concentrations of substrate 18:0-ACP from 1μM to 50μM and 400μM of NADPH were added to buffer (Buffer used: 50mM HEPES, pH 7.8, 50nM NaCl) to

make up a total reaction volume of 500µl. Concentrations of ferredoxin, ferredoxin reductase, Δ9D, oxygen and NADPH were kept constant while the concentration of the substrate acyl-ACP was varied. All components except NADPH were mixed together at room temperature, and then NADPH was added to start the reaction. In cases where k_{cat} was lower, the concentrations of protein components were increased so that there will be greater product formation with time. Reactions were quenched at various time intervals by withdrawing 200uL of the reaction and mixing rapidly with the same volume of tetrahydrofuran. The quenched reactions were then diluted with deionized water and the fatty acyl chain cleaved from ACP by addition of ~5mg of $NaBH_4$. After incubation at 37°C for 10 minutes, the remaining $NaBH_4$ was quenched by addition of 100µl of 1N HCl and then the sample converted to alkaline pH by adding 30µl of 10N NaOH. The fatty alcohols were then obtained with 2 equal volume extractions with $CHCl_3$. The pooled CHCl3 were evaporated to dryness and resuspended 100µl of hexane. 2.5µl of N-methyl-N-trimethylsilyltrifluroacetamide (MSTFA) was added to convert the fatty alcohols to silyl ether derivatives for gas chromatographic analysis[5]. The products were identified and quantitated using a GC/MS (Hewlett Packard GC 6869 series) connected to either a flame ionization detector or electron ionization mass-sensitive detector with helium used as the carrier gas.

Stopped flow spectrophotometry

The rate of formation and decay of the peroxo intermediate was monitored by stopped-flow spectrophotometry. In this experiment, approximately 500µM of Δ9D and a stoichiometrically equivalent amount of 18:0-ACP were mixed and made anaerobic,

before being reduced by dithionite. The reduced Δ9D-ACP complex was rapidly mixed observing the formation and decay of the spectra from 450-650 nm.

Results

Steady State Catalysis Assays

The K_M and k_{cat} parameters obtained from steady state catalysis assays are summarized below:

Mutant	Cloned	Expressed	Purified	Activity Assay	
				K_m (uM)	k_{cat} (min^{-1})
T199A	√	√	√	0.8	0.7
T199N	√	-	-	-	-
T199S	√	√	√	35.6	0.4
T199V	√	√	√	6.6	0.5
T199Q	√	√	√	-	-
T199D	√	√	√	2.2	0.1
T199E	√	√	√	14.7	0.2
WT	-	-	-	3.3	33

Table 1. Values of *Km* and *kcat* of T199 mutants obtained from steady state catalysis assays. The parameters of wild type Δ9D are used as comparison[5]. '√' indicates the process has been successfully completed. ' - ' indicates pending work.

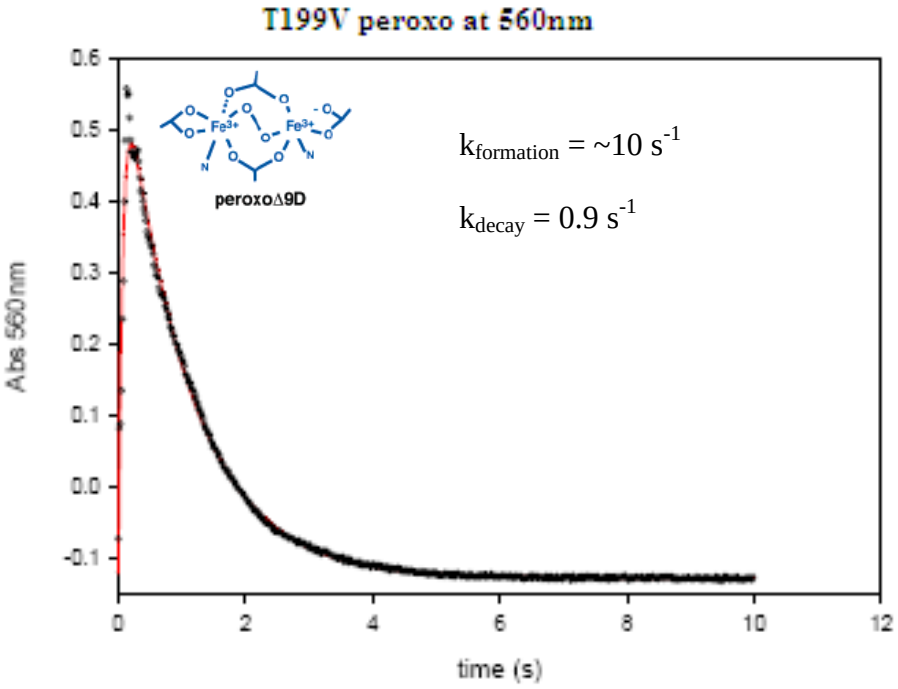
All the variants of the mutants were successfully cloned and the mutations verified through sequencing. Except for T199N, all were expressed and purified using protocols described above and concentrated to a concentration suitable for conducting steady-state catalysis assays (~100 to 400μM).

Steady state catalysis assays were done on all mutants except for T199N and T199Q. Initial desaturation velocities were determined by linear least-squares fitting of the increase of product over 3 minutes. Steady state kinetic parameters k_{cat} and K_M were determined by non-linear least-squares fitting of the initial desaturation velocities and substrate concentrations to the Michaelis-Menten equation, $\upsilon = \frac{k_{cat}[S]}{K_M + [S]}$.

For all the mutant Δ9D assayed, the k_{cat} were much lower than the wild type and showed ~100-1000 fold decrease in desaturase activity, indicating loss of activity due to

mutation. On the other hand, the K_M of mutant Δ9D showed variable changes. Except for T199A and T199D, all of the mutants showed an increase in K_M, suggesting that these mutants have a lower affinity for the substrate than the wild type enzyme.

Stopped Flow Spectrophotometry



Graph 1. Formation and decay of T199V peroxo intermediate at 560 nm. Peak of graph corresponds to peroxoΔ9D, and decrease in absorbance shows its decay. The rate constants for its decay and formation are ~10s^{-1} and 0.9s^{-1} respectively.

Studies were done using T199V. The $t_{1/2}$ of the peroxo intermediate decay is less than a minute. The peroxo intermediate formed by the mutant T199V is extremely short-lived compared to wild type Δ9D which has a $t_{1/2}$ of about 26 minutes. Data was fit to a sequential exponential sequence of A→B→C to obtain rate constants of $k_{formation}$ = ~10 s^{-1} and k_{decay} = 0.9 s^{-1}.

Discussion

The mutants displayed ~100-1000 fold decrease in desaturase activity, as shown in the lower rate constants. This is expected and indicates that Threonine 199 plays a role in catalysis; change of the amino acid residue causes a loss or decrease in activity. The activity of T199D is consistent with previously reported values[4], while the activity of T199E is higher than reported. Disparity between these values and previous values could be due to the different methods of workup used or experimental error. Assays will be repeated to ensure experimental errors are reduced.

At the 199th position in the wild type Δ9D, threonine is a polar uncharged residue. Through the mutations that were designed, threonine has been changed to negatively charged groups (T199D, T199E), non-polar groups (T199A, T199V) or polar uncharged residues with a smaller side chain (T199S). All mutations showed a drastic decrease in the catalytic activity. When side chains are changed to non-polar or negatively charged, the decrease in activity is expected as the property of the residue has changed.
As Threonine 199 is a "second sphere" residue, it is likely responsible for protein interactions and positioning of other residues in the active site; by changing its properties, this residue is unable to function as effectively. When the size of the side chain of the 199th residue is reduced in T199S, there was also a drop in the activity of the enzyme. The decrease in the activity of T199S indicates that the size of the side chain is also important in the functioning of the 199th residue.

The specificity constants, K_m, of the mutants were variable. Since K_m indicates the affinity of the enzyme for the substrate, T199S, T199V and T199E having higher K_m constants indicate that they have a lower affinity of the enzyme, and this is expected since the property of the residue at the 199th position has been changed. T199A and T199D have lower K_m, indicating that they have a higher affinity for the substrate than the wild type enzyme. However, their rate constants are still lower than the wild type enzyme. This suggests that T199A and T199D might be binding better to the substrate, but the reaction is not catalyzed at the same rate as that observed in the wild type.

Studies done using T199V in stopped flow spectrophotometry showed that the peroxo intermediate formed quickly and decayed much faster than the peroxo intermediate formed in the wild type Δ9D ($t_{1/2}$ ~26min). Because the property of the residue at 199th position has changed in the mutants, the faster rate of decay confirms that Threonine 199 contributes to stabilizing the peroxo intermediate formed during turnover of chemically reduced Δ9D and may provide insight into stabilization of the intermediate during catalysis. Stopped-flow spectrophotometric assays will also be done on other mutants to examine the stability of the peroxo intermediates formed.

Studies of Threonine 199 done in Δ9D can be relevant to studying similar diiron binding motifs in other enzymes, such as other desaturases, R2 subunit of the ribonucleotide reductase and bacterial multicomponent monooxygenases. A similarly situated threonine residue in cytochrome P450 is thought to play a role in proton transfer during catalysis. The evolutionarily related bacterial multicomponent monooxygenases

possess a conserved threonine in a similar position relative to the active site[1]. Recent structural studies from our lab have indicated a role for this residue in positioning both a second sphere glutamine and an active site glutamate during catalysis. A similar bonding network may be formed during catalysis in Δ9D upon formation of the protein-protein interactions required in the reaction cycle. Further work on the multi-protein complexes of Δ9D will be required to elucidate the structural determinants of catalysis.

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