Azide and Acetate Complexes Plus Two Iron-depleted Crystal Structures of the Di-iron Enzyme Δ9 Stearoyl-Acyl Carrier Protein Desaturase

IMPLICATIONS FOR OXYGEN ACTIVATION AND CATALYTIC INTERMEDIATES

Received for publication, February 17, 2003, and in revised form, April 14, 2003
Published, JBC Papers in Press, April 18, 2003, DOI 10.1074/jbc.M301662200

Martin Moche‡, John Shanklin§, Alokesh Ghoshal‡, and Ylva Lindqvist‡‡

From the ‡Department of Medical Biochemistry and Biophysics, Karolinska Institutet, Stockholm S-171 77, Sweden and the §Department of Biology, Brookhaven National Laboratory, Upton, New York 11973

Δ9 stearoyl-acetyl carrier protein (ACP) desaturase is a μ-oxo-bridged di-iron enzyme, which belongs to the structural class I of large helix bundle proteins and that catalyzes the NADPH and O2-dependent formation of a cis-double bond in stearoyl-ACP. The crystal structures of complexes with azide and acetate, respectively, as well as the apo- and single-iron forms of Δ9 stearoyl-ACP desaturase from Ricinus communis have been determined. In the azide complex, the ligand forms a μ-1,3-bridge between the two iron ions in the active site, replacing a loosely bound water molecule. The structure of the acetate complex is similar, with acetate bridging the di-iron center in the same orientation with respect to the di-iron center. However, in this complex, the iron ligand Glu196 has changed its coordination mode from bidentate to monodentate, the first crystallographic observation of a carbonato shift in Δ9 stearoyl-ACP desaturase. The two complexes are proposed to mimic a μ-1,2 peroxo intermediate present during catalytic turnover. There are striking structural similarities between the di-iron center in the Δ9 stearoyl-ACP desaturase-azide complex and in the reduced ruberythrin-azide complex. This suggests that Δ9 stearoyl-ACP desaturase might catalyze the formation of water from exogenous hydrogen peroxide at a low rate. From the similarity in iron center structure, we propose that the Δ9 stearoyl-ACP desaturase might be an enzyme that can oxidize the iron center in the same orientation with respect to the di-iron center as in ruberythrin and not as reported for the R2 subunit of plastid catalyzing the NADPH- and O2-dependent insertion of a cis-double bond between the C-9 and C-10 positions of saturated fatty acids (1–3). The natural substrate, stearic acid, is attached to acyl carrier protein (ACP) via a thioester linked to a pantetheine group (4). Desaturase isozymes act specifically on saturated fatty acids of varying chain length and differ in the insertion position of the double bond (5–9). The enzymes interact directly with molecular oxygen and reduced ferredoxin during catalysis, with the electrons ultimately coming from NADPH via ferredoxin reductase or directly from photosystem I (10).

Δ9 Desaturase is a homodimer with each mature subunit of 41.6 kDa containing a binuclear iron site (11). It belongs to the structural class I of large helix bundle proteins, which also includes the R2 subunit of ribonucleotide reductase (R2) and the hydroxylating subunit of methane monoxygenase (MMOH) (12). Both oxidized and fully reduced Δ9 desaturase have been characterized spectroscopically (11, 13, 14). It was shown that the oxidized iron center contains a μ-oxo bridge, which is lost upon reduction, in agreement with the crystal structure of photoreduced Δ9 desaturase, determined to 2.4-Å resolution (15).

The Δ9 desaturase monomer consists mainly of α-helices with the catalytic di-iron center buried within a four-helix bundle. Two pairs of anti-parallel helices provide ligands to the iron ions: α2-Glu155 plus α4-Glu143 and -His146 and α2-Glu196 plus α4-Glu220 and -His232 (Fig. 1). The distance between the two 5-coordinated iron ions is 4.1 Å, and they have distorted square pyramidal coordination geometry. The structure of the cluster is highly symmetric. Glu155 is a bidentate ligand to one iron ion (Fe1), and correspondingly, Glu196 is a bidentate ligand to the second iron ion (Fe2). Glu143 and Glu220 both act as bridging ligands of the iron center. Besides these carbohydrate ligands, each iron ion has one nitrogen atom ligand, N61 of His146 and His232, respectively. A water molecule is loosely coordinated to the iron center at a distance of 3.0 and 3.3 Å to the Fe1 and Fe2 ions, respectively. A narrow, bent, hydrophobic cavity, expected to bind the saturated fatty acid substrate, extends from the surface down into the protein for −20 Å. Where it passes the di-iron cluster, the shape favors a gauche substrate conformation, which predisposes the formation of a cis-double bond in the product. Based on this crystal structure,
the substrate fatty acid chain length specificity was altered in desaturase by making series of mutations guided by the structure (16).

The catalytic mechanism of soluble desaturases remains to be fully elucidated but is expected to have intermediates in common with other di-iron enzymes of class I, especially methane monooxygenases, since these can catalyze desaturase reactions with some substrates (17). To react with molecular oxygen, the resting ferric di-iron center needs to be reduced by ferrodoxin in two single-electron transfer steps. Δ9 desaturase has not been observed in a one-electron reduced form, so the addition of the first electron seems rate-limiting for reduction (11). The binding surface on Δ9 desaturase for ferrodoxin remains to be determined. Reaction of the reduced ferrous center with molecular oxygen gives rise to a peroxo intermediate. This is followed by a highly reactive intermediate, able to abstract hydrogen atoms from the saturated fatty acid bond in the cavity, which has been proposed to be similar to the ferryl “Q” intermediate in MMOH (18). However, since the outcome of the reaction with natural substrates is different for Δ9 desaturase and MMOH, desaturation versus hydroxylation, this hypothesis remains to be tested. Modeling stearic acid in the hydrophobic cavity of Δ9 desaturase places the C-9 carbon in the hydrophobic cavity and the C-10 carbon atom near the di-iron center. Recent data give some indication that the initial hydrogen abstraction takes place at carbon 10 (19, 20). However, the detailed mechanism for the desaturation remains to be elucidated.

A stable peroxo intermediate can be obtained by mixing chemically reduced Δ9 desaturase with stearoyl-ACP under anaerobic conditions and then exposing the sample to one atmosphere of O2 (21,22). Chemical reduction with dithionite reduces both subunits of the desaturase dimer in a 4e reduction in contrast to the biological 2e reduction, where one subunit of Δ9 desaturase is reduced at a time. This peroxo-diferric intermediate of desaturase is more stable than similar peroxo intermediates of either MMOH or of mutants of R2 (23–26). It decays without formation of the product oleoyl-ACP or of hydrogen peroxide but through an oxidative reaction forming water (22).

We have now determined the structures of an azide complex of Δ9 desaturase to 2.4 Å resolution and an acetate complex to 2.4 Å resolution. We propose that these azide and acetate com-

\[
\text{Fe}^2+ + \text{O}_2 + \text{H}_2\text{O} \rightarrow \text{Fe}^3+ + \text{O}_2^{-} + \text{H}^+ + \text{OH}^{	ext{-}}
\]

bining bonds are shown as dotted lines. The view is equivalent to the view in Figs. 2, 4, 5, and 6.

MATERIALS AND METHODS

Protein Preparations—The mature castor protein, lacking the 33-amino acid transit peptide, was expressed under the control of the T7 promoter in Escherichia coli strain BL21 gold (Novagen, Madison, WI). Cells were grown in a Bioflow 3000 fermenter (New Brunswick Scien-

The Di-iron Center of Δ9 Stearoyl-ACP Desaturase

fig. 1. Stereoview of the di-iron center of reduced desaturase (1AFR) determined at 2.4 Å. Iron center coordi-

the view is equivalent to the view in Figs. 2, 4, 5, and 6.

tific, New Brunswick, NJ), in Luria-Bertani broth supplemented with 1% (w/v) glucose to an A_{600} of ~3 at which time the cells were induced by adding lactose to 0.4% (w/v) and incubating for 4 h at 30 °C. Dis- 

solved oxygen was maintained above 15% and pH was maintained at 6.5. Cell densities at harvest were ~12 A_{600}. Cells were resuspended 1:2 (w/v) in 50 mM HEPES, 2 mM phenylmethylsulfonyl fluoride, pH 7.5, containing 1 mg/ml fresh weight of DNAse I and were disrupted by passage through a French pressure cell with a 70-megapascal pressure drop. The lysate was clarified by centrifugation at 250,000 × g for 30 min and applied to a Poros20CM column (Foster City, CA) developed with a linear gradient of 20 column volumes of 20 mM HEPES, pH 7.5, containing 0–600 mM NaCl. Fractions enriched in desaturase were identified by SDS-PAGE, pooled, and concentrated with the use of an Amicon PM30 ultrafilter. The concentrate was subjected to size exclusion chromatography with a TSK G3000SW column (Mac Mod Analytical, Chadds Ford, PA) developed with 20 mM HEPES, pH 7.0, 70 mM NaCl. Desaturase enriched fractions were identified by SDS-PAGE and concentrated as before to ~0.15 mM desaturase (dimer) prior to crystallization.

Crystallizations—The hanging drop vapor diffusion method has been used for all crystallization experiments. The azide complex was prepared by adding 70 mM sodium azide to the protein solution prior to crystallization. Crystals were obtained in 0.08 M cacodylate buffer, pH 5.4, 200 mM magnesium acetate, 75 mM ammonium sulfate, 0.2% octyl glucoside, and 12–15% polyethylene glycol 4000 as precipitant as published for holoenzyme (15). The crystals were cryoprotected by a short soak in well solution with water exchanged for 20% (v/v) 2-methyl-2,7-

pentandiol. The cell parameters were determined with the CCP4 suite (30).

Structure Determination and Refinement—All structures were solved by guest on December 21, 2017 http://www.jbc.org/ Downloaded from by guest on December 21, 2017
**The Di-iron Center of Δ9 Stearoyl-ACP Desaturase**

**Table I**

<table>
<thead>
<tr>
<th>Data collection and structure refinement</th>
<th>Iron-free</th>
<th>One iron</th>
<th>Acetate complex</th>
<th>Azide complex</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Space group</strong></td>
<td>P3&lt;sub&gt;1&lt;/sub&gt;</td>
<td>P&lt;sub&gt;2&lt;/sub&gt;&lt;sub&gt;1&lt;/sub&gt;&lt;sub&gt;2&lt;/sub&gt;&lt;sub&gt;1&lt;/sub&gt;</td>
<td>P&lt;sub&gt;3&lt;/sub&gt;&lt;sub&gt;1&lt;/sub&gt;&lt;sub&gt;2&lt;/sub&gt;</td>
<td>P&lt;sub&gt;2&lt;/sub&gt;&lt;sub&gt;1&lt;/sub&gt;&lt;sub&gt;2&lt;/sub&gt;&lt;sub&gt;1&lt;/sub&gt;</td>
</tr>
<tr>
<td><strong>Mol/ASU</strong></td>
<td>6</td>
<td>6</td>
<td>1</td>
<td>6</td>
</tr>
<tr>
<td><strong>Cell axis a</strong></td>
<td>188.0</td>
<td>192.4</td>
<td>94.1</td>
<td>192.9</td>
</tr>
<tr>
<td><strong>Cell axis b</strong></td>
<td>188.0</td>
<td>145.8</td>
<td>94.1</td>
<td>145.2</td>
</tr>
<tr>
<td><strong>Cell axis c</strong></td>
<td>82.1</td>
<td>81.9</td>
<td>81.7</td>
<td>81.8</td>
</tr>
<tr>
<td><strong>Resolution</strong></td>
<td>3.2 (3.3-3.2)</td>
<td>2.8 (2.85-2.8)</td>
<td>2.4 (2.53-2.4)</td>
<td>2.4 (2.46-2.4)</td>
</tr>
<tr>
<td><strong>R&lt;sub&gt;sym&lt;/sub&gt;</strong></td>
<td>0.065 (0.196)</td>
<td>0.058 (0.252)</td>
<td>0.043 (0.283)</td>
<td>0.057 (0.299)</td>
</tr>
<tr>
<td><strong>R&lt;sub&gt;f&lt;/sub&gt;</strong></td>
<td>12.2</td>
<td>12.1</td>
<td>13.5</td>
<td>14.8</td>
</tr>
<tr>
<td><strong>Completeness</strong></td>
<td>92.7 (65.1)</td>
<td>79.3 (32.0)</td>
<td>99.4 (98.9)</td>
<td>99.3 (97.9)</td>
</tr>
<tr>
<td><strong>Reflections (%) with I&lt;sub&gt;f&lt;/sub&gt; &gt; 3.0</strong></td>
<td>69.8 (22.9)</td>
<td>44.3 (10.0)</td>
<td>82.4 (50.3)</td>
<td>80.3 (47.5)</td>
</tr>
<tr>
<td><strong>Refinement</strong></td>
<td>REFMAC5</td>
<td>REFMAC5</td>
<td>REFMAC5</td>
<td>REFMAC5</td>
</tr>
<tr>
<td><strong>Reflections working set</strong></td>
<td>47,756</td>
<td>44,537</td>
<td>15,426</td>
<td>87,879</td>
</tr>
<tr>
<td><strong>Reflections test set</strong></td>
<td>1471</td>
<td>935</td>
<td>819</td>
<td>2236</td>
</tr>
<tr>
<td><strong>R-work (%)</strong></td>
<td>22.8</td>
<td>22.4</td>
<td>21.6</td>
<td>22.9</td>
</tr>
<tr>
<td><strong>R-free (%)</strong></td>
<td>25.7</td>
<td>24.8</td>
<td>26.4</td>
<td>24.1</td>
</tr>
<tr>
<td><strong>Atoms modeled</strong></td>
<td>16,848</td>
<td>16,847</td>
<td>2822</td>
<td>17,235</td>
</tr>
<tr>
<td><strong>Number of iron</strong></td>
<td>0</td>
<td>6</td>
<td>2</td>
<td>12</td>
</tr>
<tr>
<td><strong>Number of strontium</strong></td>
<td>12</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><strong>Number of water</strong></td>
<td>0</td>
<td>5</td>
<td>73</td>
<td>368</td>
</tr>
<tr>
<td><strong>Deviation from ideals (root mean square)</strong></td>
<td>0.016</td>
<td>0.013</td>
<td>0.009</td>
<td>0.010</td>
</tr>
<tr>
<td><strong>Bonds (degrees)</strong></td>
<td>1.52</td>
<td>1.24</td>
<td>1.02</td>
<td>1.12</td>
</tr>
<tr>
<td><strong>Ramachandran plot</strong></td>
<td>87.9</td>
<td>88.6</td>
<td>90.6</td>
<td>89.9</td>
</tr>
<tr>
<td><strong>Most favored</strong></td>
<td>87.9</td>
<td>88.6</td>
<td>90.6</td>
<td>89.9</td>
</tr>
<tr>
<td><strong>Additional allowed</strong></td>
<td>11.8</td>
<td>10.8</td>
<td>8.8</td>
<td>9.8</td>
</tr>
<tr>
<td><strong>Generously allowed</strong></td>
<td>0.0</td>
<td>0.3</td>
<td>0.3</td>
<td>0.0</td>
</tr>
<tr>
<td><strong>Disallowed</strong></td>
<td>0.3</td>
<td>0.3</td>
<td>0.3</td>
<td>0.3</td>
</tr>
</tbody>
</table>

---

**RESULTS**

**Crystal Packing and Space Groups**—Crystals from three space groups, P<sub>2</sub><sub>1</sub><sub>2</sub><sub>1</sub>, P<sub>3</sub><sub>1</sub>, and P<sub>3</sub><sub>1</sub><sub>2</sub>, with approximately the same length of their shortest cell axis have been obtained during this work (Table I). During crystallization, the desaturase dimers have a strong tendency to pack along a 3-fold screw axis, being noncrystallographic in the P<sub>2</sub><sub>1</sub><sub>2</sub><sub>1</sub> crystal form, and are described in more detail in the original structure determination. The packing of desaturase dimers along the 3-fold axis gives rise to “rodlike” structures, and the three different space groups are created when these rods pack differently with respect to each other.

**Quality of the Models**—No large conformational changes are observed for the four models presented here compared with the original model (1AFR) of Δ9 desaturase determined at room temperature (Table I). Minor differences are localized to some remaining positive electron density isobserved in some areas where all of the structures show very high B-factors and in some regions badly defined electron density. These areas comprise residues 18–50, residues 205–215, and in particular residues 338–346, which have been omitted in the acetate complex in P<sub>3</sub><sub>1</sub>, where they appear disordered. In regions of badly defined electron density, side chain occupancies have been set to zero.

For the azide complex P<sub>2</sub><sub>1</sub><sub>2</sub><sub>1</sub> and the acetate complex in P<sub>3</sub><sub>1</sub>, the electron density maps are of excellent quality in the active site. However, due to the absence of iron ions, the two iron-depleted models show considerable disorder of the carboxylate side chains in the active site. In all structures, the iron center bridging Glu<sup>229</sup> has more flexibility than other iron center ligands, as evidenced by higher B-factor and less well defined electron density.

**Azide Binding to the Δ9 Desaturase Di-iron Center**—Occupancy of azide in the desaturase active site was high, and difference electron density corresponding to the azide ion was found in all six subunits of the asymmetric unit. No significant structural shifts have been introduced upon azide binding. The azide ion bridges the two iron ions of the desaturase di-iron center with no significant changes in the geometry of the coordinating carboxylate or histidine ligands compared with the original structure of the ferrous di-iron center. Therefore, this structure most likely represents azide bound to the reduced state of desaturase with an iron-iron distance of 4.1 Å. The azide ion binds the iron cluster in a μ-1,3 bridging mode, with iron-nitrogen distances of 2.5–2.6 Å (Fig. 2). Azide has displaced the weakly coordinated water molecule axial to the histidines, and the carboxylates are left in the plane of a distorted octahedron (Figs. 1 and 2). The orientation of the azide ion is clearly defined in the 2F<sub>o</sub> − F<sub>c</sub> electron density map, but some remaining positive electron density is observed in some...
subunits in the \( F_o - F_c \) map. This could reflect a small population of a second azide conformation that differs from that modeled in our 2.4-Å electron density map.

A pathway of eight water molecules (four from each subunit) across the dimer interface connects the iron centers, which are \(-23.5\) Å apart (Fig. 3a). These water molecules form hydrogen bonds to main and side chain residues of the protein, and they are well defined in our electron density maps; three of them were also present in the original holo structure. The distance between the azide and the closest water molecule in the pathway is \(3.6\) Å. This water channel is present in both the azide-P2\(_{1}2\(_{1}2\(_{1}\) and the acetate-P3\(_{1}12\) structure.

**Acetate Binding to the \( \Delta^9 \) Desaturase Di-iron Center**—The overall structure of desaturase is very similar between this P3\(_{1}12\) structure and the P2\(_{1}2\(_{1}2\(_{1}\) original structure (15). The root mean square deviation of \(0.50\) Å for 337 C-\(\alpha\) carbon atoms (Table II) is only slightly higher than for the P2\(_{1}2\(_{1}2\(_{1}\) azide complex, \(0.36\) Å over 345 C-\(\alpha\) atoms. There is a very slight reorientation of the subunits in the dimer in this space group; alignment of the dimer to the original holo structure gives a root mean square value of \(0.66\) Å versus \(0.39\) Å for the dimer azide complex.

Since the distance between the iron ions is long, \(4.0\) Å, we presume that the metal center was reduced during x-ray data collection as was previously reported for the holodesaturase crystals. Acetate bridges the iron center, displacing a water molecule, weakly coordinating the two irons in the structure of the reduced enzyme. The oxygen to iron distance is \(2.6\) and \(2.5\) Å, respectively. When acetate binds to the desaturase di-iron center (Fig. 4), Glu\(_{196}\), a bidentate ligand in the original structure of the reduced di-iron center (Fig. 1), appears as a monodentate ligand to Fe2. The distance between Fe2 and the oxygen atoms of Glu\(_{196}\) are unchanged for Oe1 (2.2 Å), whereas Oe2 increases from 2.5 to 3.0 Å between the structures. The 2.4-Å electron density map of the acetate complex is of high quality in the active site, and the small shift observed in Glu\(_{196}\) is therefore considered a valid feature of the structure. The orientation of Glu\(_{196}\) was confirmed by calculation of an annealed omit map, and furthermore, the shift is reproducibly obtained on refinement after perturbation of the side chain to its bidentate configuration. Acetate binding in conjunction with this carboxylate shift results in a distorted octahedral coordi-
nation of Fe1 (five protein ligands plus one acetate ligand) and a distorted trigonal bipyramidal coordination of Fe2 (four protein ligands and one acetate ligand).

The One Iron Structure of \( \Delta 9 \) Desaturase—The electron density maps showed unambiguously that one of the iron ions had been lost from the cluster at pH 4. Fe1 remains in its position in the active site with uncharged 5-coordination by ligands from the protein, whereas Fe2 is lost to the surrounding solvent (Fig. 5). No large structural perturbations are observed. Compared with the original ferrous di-iron center structure (Fig. 1), the absence of Fe2 causes some flexibility in its bidentate ligand Glu196 and in the normally bridging Glu229. The positions of the other two carboxylate iron ligands, Glu105 and Glu143, are not changed compared with the reduced di-iron center. Glu229 and Glu196 appear to form a 2.6-Å hydrogen bond between each other, probably caused by protonation of Glu196 Oe2 at pH 4. We are unable to distinguish whether the remaining Fe1 is in the ferrous state or in the oxidized ferric state.

The Structure of Apo \( \Delta 9 \) Desaturase—Electron density for the iron cluster was completely absent in this structure and the normally liganding residues showed some disorder. The overall structure of the apo form of \( \Delta 9 \) desaturase shows little difference to the ferrous enzyme at this resolution. The protein is thus able to form a stable structure also in the absence of the positive charges provided by the di-iron center. The buried carboxylate side chains of the empty di-iron center shift positions slightly and appear to be protonated, since they are at hydrogen bond distance to each other (Fig. 6); a similar observation was made for the apo form of R2 (40). The adjacency of the carboxylate side chains has increased their \( pK_a \), allowing them to be protonated at pH 6.7. In the absence of protonation of the di-iron ligands, the close proximity of negative charges would be expected to destabilize the desaturase four-helix bundle. The resulting pattern of hydrogen bonds could be different in the ensemble of protein molecules in the crystals and contributes to the observed disorder of the carboxylate residues in the di-iron center, especially Glu229, which is almost undefined in the 2\( F_o \) – \( F_e \) electron density map.

A peak of very high difference electron density, probably representing a novel metal ion-binding site, was found in the dimer interface of apodesaturase (Fig. 3b). Since strontium chloride (60 mM) was present during crystallization, we expect the bound metal ion to be strontium. We have observed the same metal-binding site but with bound iron ions in electron density maps of other desaturase crystals.\(^2\) The metal ion has bidentate coordination by Glu196 from both subunits of the dimer at distances in the range of 2.8–3.2 Å. There are no water molecules modeled in this low resolution map of apodesaturase, but the metal in the dimer interface is located in the middle of the pathway of water molecules connecting the two metal centers of the \( \Delta 9 \) desaturase dimer (Fig. 3c). The distance from the high affinity iron (Fe1) sites and the iron in the dimer interface is 12.2 Å.

DISCUSSION

The Azide Complex—Earlier spectroscopic studies have shown that when azide binds the resting oxidized state of \( \Delta 9 \) desaturase, the \( \mu \)-oxo bridge is either protonated or lost (41). Spectroscopy further predicted two different, pH-dependent, binding modes of azide (\( \mu \)-1,3-bridging and \( \eta^1 \)-terminal), with \( \sim90\% \) being in the bridging mode at pH 6.2, which is the pH used in our crystallization. The crystal structure of the ferrous azide complex shows the \( \mu \)-1,3 binding mode but also shows some difference density, suggesting the presence of a small proportion of azide in a \( \mu \)-1,1 binding mode. In fact, the spectroscopic data for the second conformation of the ferric azide complex could be interpreted as either \( \eta^1 \)-terminal with a protonated \( \mu \)-oxo bridge or as \( \mu \)-1,1 coordination, where the \( \mu \)-oxo bridge is lost. The data presented here support the latter model.

No structures of azide complexes from wild-type di-iron center enzymes of this class are known. However, they have been determined for the F208A/Y122F and E238A/Y122F mutants of the reduced R2 subunit of ribonucleotide reductase (42, 43). The azide ion is close to \( \mu \)-1,1-bridging in both R2 mutants occupying the position of the \( \mu \)-oxo bridge (E238A/Y122F) or of a second solvent molecule (F208A/Y122F) coordinated by Fe2 in the oxidized di-iron center. In both mutants of R2, the azide extends away from the di-iron center, occupying the available space introduced by the mutations.

In contrast to these R2-mutant complexes, the desaturase azide complex is strikingly similar to the azide complex of reduced rubrerythrin (Fig. 7), showing virtually identical coordination (44). As in desaturase, binding of azide to the reduced state of rubrerythrin introduces no carboxylate shifts in the surrounding iron ligands, and it binds azide in the same fash-
An important difference in the structure of the oxidized iron center in rubrerythrin and in oxidized R2 and MMOH is the position of the μ-(hydr)oxo bridge (45–47). Whereas it replaces the position corresponding to Glu 229 in reduced R2 and MMOH, it is at the other side of the iron-iron axis (where MMOH has its second solvent bridge) in rubrerythrin, occupying the site where azide is bound. From spectroscopy, the μ-oxo-bridge of azide is known to displace the μ-oxo-bridge in desaturase, and we thus suggest that the μ-oxo-bridge in oxidized desaturase is bound similarly as in rubrerythrin.

The Acetate Complex—Carboxylate shifts have been proposed to play an essential role during catalysis in di-iron enzymes through modifying the electronic structure of the iron center (48). The small carboxylate shift of Glu196 observed in the acetate complex changes the low affinity iron ion (Fe2) geometry from a distorted octahedral coordination to a distorted trigonal bipyramidal coordination. This represents the first carboxylate shift identified in a Δ9 desaturase crystal structure.

In a study of magnetic circular dichroism in chemically reduced Δ9 desaturase, it has been shown that the coordination environment of the reduced di-iron center is influenced by the presence of stearoyl-ACP, changing it from two equivalent 5-coordinated irons in a distorted square pyramidal geometry into a geometry where Fe2 is 4-coordinated (49). This correlates to what is observed here, where both iron ions of the original reduced di-iron center are 5-coordinated, but in the acetate complex, Fe2 is 4-coordinated by ligands from the protein. It was suggested that the change in iron coordination, caused by binding of stearoyl-ACP, increases the reactivity against molecular oxygen (49). This is an attractive model, since it would prevent formation of reactive oxygen intermediates in the absence of stearoyl-ACP. In our case, the reactivity of the di-iron center toward binding of acetate is indeed increased by this change in coordination, since acetate does not bind to the di-iron center in P212121 but only to the di-iron center in the P3121 crystal form, where Fe2 is 4-coordinated. No significant shift in ligation is observed in the azide complex in P212121.

Acetate has been observed bound to the oxidized di-iron center of methane monooxygenase hydroxylase from M. capsulatus (47), and the two complexes are compared in Fig. 8. Acetate binds in the same location opposite to the histidine iron ligands. In the case of Δ9 desaturase, we suggest that acetate has replaced the μ-oxo bridge, but in MMOH it has replaced a second solvent bridge. In both Δ9 desaturase and MMOH, the histidine iron ligands are hydrogen bonding to carboxyl side chains. Conserved aspartate residues in di-iron hydroxylases (50) are structurally equivalent to Asp228 and Glu142 in Δ9 desaturase. In both desaturases and hydroxylases, the charges of these carboxylate side chains are compensated for by interaction with conserved arginine residues corresponding to Arg231 and Arg145 in Δ9 desaturase. The difference is that the histidine ligand of the low affinity Fe2 forms a hydrogen bond to a conserved glutamate (Glu142) in Δ9 desaturase and a
The conserved Glu97 of rubrerythrin. Sequence-conserved Thr\textsuperscript{199} of desaturase corresponds to the conserved Glu\textsuperscript{97} of rubrerythrin.

Conserved aspartate (Asp\textsuperscript{143}) in MMOH (Fig. 8). The conservation of these histidine-liganding carboxylates underscores the importance of correct positioning of the histidines for the geometry and reactivity of the di-iron clusters.

Azide- and Acetate-bound Iron Centers as Analogues of a Peroxodiferric Intermediate of Δ9 Desaturase—A stable μ-1,2-peroxo intermediate species that decays with formation of water instead of oleoyl-ACP can be prepared (21, 22). The azide bound with μ-1.3 coordination to the iron and the similarly bound acetate are assumed to mimic this unreactive intermediate. The formation of water instead of product from this stable peroxide intermediate is probably caused by an excess of electrons present due to chemical reduction. The extra electrons delivered from surplus dithionite or from the second subunit of desaturase can intercept formation of the reactive intermediate that follows the peroxo intermediate, preventing it from abstracting hydrogens from stearic acid. An attractive hypothesis is that the extra electrons are delivered via the water pathway that connects the two iron sites and that the required protons are delivered via the same path (i.e., a net transfer of two hydrogens).

Little information about the intermediates formed in the presence of the natural electron transport chain is available for Δ9 desaturase, since most of the results obtained from spectroscopy have been made with chemically reduced enzyme. When the natural electron transport chain is used for catalysis, the peroxo intermediate, leading to formation of oleoyl-ACP, is short lived in contrast to the μ-1,2 peroxide intermediate that decays with formation of water (22). The subtle structural modifications giving rise to this difference in reactivity are unknown, but it seems reasonable to assume that the stable peroxo intermediate and the catalytically competent peroxo intermediate will be quite similar. We propose that the acetate intermediate will be quite similar. We propose that the acetate intermediate introduces and expressed in yeast (C. elegans) increased the tolerance against hydrogen peroxide to Δ9 desaturase in the yeast, although this was attributed to changes in membrane fluidity and not to peroxidase activity of the introduced Δ12 desaturase gene (53).

Iron-deficient Δ9 Desaturase—Since desaturase can be observed with only one of the iron sites specifically occupied at pH 4, the two binding sites must have different affinities for iron. This is a reflection of differences in pK\textsubscript{a} of the carboxylate complex. The nature of the reactive intermediate that follows the peroxo intermediate in desaturase and abstracts hydrogen atoms from stearic acid remains to be characterized.

The reaction that follows upon the addition of hydrogen peroxide to Δ9 desaturase is currently not well documented, but it is known that the presence of catalase speeds up the desaturase reaction in the enzymatic assay (3, 51). The hydrogen peroxide consumed by catalase was suggested to be formed by ferredoxin and ferredoxin reductase used in the desaturase assay (3). We propose that hydrogen peroxide inhibits Δ9 desaturase by binding to the reduced di-iron center bridging the iron atoms in the same way as the azide and acetate ions presented here. Taken together, the striking similarity of the Δ9 desaturase and rubrerythrin azide complexes and the ability of the μ-1,2 peroxodiferric intermediate in Δ9 desaturase to decay with formation of water, raise the possibility that the reduced state of desaturase can act as a peroxidase, albeit at a low rate. Formation of water is slow, since a residue corresponding to Glu\textsuperscript{97} of rubrerythrin, catalyzing the required proton transfer (44, 52), is occupied by Thr\textsuperscript{199} in Δ9 desaturase. Experiments are currently under way to test this hypothesis.

If desaturases have a low peroxidase activity, they might have a secondary role contributing to protection against oxidative stress. It has been shown that a Δ12 desaturase from C. elegans introduced and expressed in yeast (Saccharomyces cerevisiae) increased the tolerance against hydrogen peroxide in the yeast, although this was attributed to changes in membrane fluidity and not to peroxidase activity of the introduced Δ12 desaturase gene (53).
The Di-iron Center of Δ9 Stearoyl-ACP Desaturase

ligands that will be manifested as a difference in affinity also at physiological pH. Fe1 is the high affinity and Fe2 is the low affinity binding site for iron in the di-iron center. Differential iron affinity has been observed for other di-iron proteins (e.g. MMOH, the R2 subunit of ribonucleotide reductase, and bacterioferritin) (54–57). Desaturase has the same high affinity site (Fe1) as MMOH from M. capsulatus and bacterioferritin from *Rhodobacter capsulatus*, whereas R2 from both mouse and *E. coli* have Fe2 as their high affinity site.

From Mössbauer spectroscopy, it is known that Δ9 desaturase, which has been reduced by dithionite, either by itself or in the presence of the substrate stearoyl-ACP, consists of two high spin ferrous iron atoms with slightly different Mössbauer parameters (21). Since no spectroscopy has been reported regarding desaturase reduced by the natural electron transport chain, the spin state of the catalytically competent di-iron center remains unknown. The high spin ferrous state has more loosely bound electrons than the low spin ferrous state; therefore, at least one of the irons could be expected to be in the high spin ferrous state when delivering the first electron to molecular oxygen. It is possible that a mixture of one ferrous iron high spin and one ferrous iron low spin is the optimal arrangement for activating molecular oxygen, since the low spin state has a higher affinity for oxygen at least in heme-containing globins (58, 59). The low spin iron could be used for binding molecular oxygen, whereas the high spin iron delivers the first electron. The high spin iron in Δ9 desaturase would be Fe2, since it is more loosely bound than Fe1, and the Fe1 would bind the oxygen.

To date, there is no evidence for a system active for iron insertion into desaturases; indeed, in overexpression of the re-combinant enzyme, iron insertion occurs spontaneously. The presence of an auxiliary binding site for metal ions at the subunit surface, as observed with a bound strontium ion in the iron-free structure and bound iron ions in other Δ9 desaturase structures, suggests a possible route for spontaneous iron insertion. Under this scenario, primary iron trapping would then be transported along the water channel connecting this site to the iron center.

**Conclusions**—The Δ9 desaturase acetate complex reveals the first observation of a carboxylate shift for a desaturase di-iron enzyme. This makes Fe2 4-coordinate with respect to protein ligands, increasing the binding affinity for acetate. The desaturase azide and acetate complexes are proposed to mimic a stable μ-1,2-peroxo intermediate closely related to the catalytically competent species present during productive turnover. The strong structural similarity to the reduced ruberythrin azide complex suggests that desaturase might catalyze formation of water from hydrogen peroxide at a low rate. We also propose that the μ-oxo bridge in oxidized Δ9 desaturase is bound as in ruberythrin rather than as in R2 and MMOH.

The structure determinations of the apo and iron forms of Δ9 desaturase show that the enzyme forms a stable structure upon removal of iron from the active site. They further show that the two iron atoms bind with different affinity to the di-iron center and identify Fe1 as the high affinity iron site.

**Acknowledgments**—We thank the staff, especially the beamline managers and supporting teams, for access to Synchrotron radiation at the NSLS, beamline X25 (Michael Becker) and X12B (Dieter Schneider) (Department of Biology, Brookhaven National Laboratory, New York) and Tynge Cerenius and supporting staff at the synchrotron in Lund, beamline 711, MAX laboratory, University of Lund. We are grateful to Martin Högberg and Pål Nordlund at Stockholm University for sharing the coordinates of the azide complex of R2 prior to deposition in the Protein Data Bank.
Azide and Acetate Complexes Plus Two Iron-depleted Crystal Structures of the Di-iron Enzyme Δ9 Stearoyl-Acyl Carrier Protein Desaturase: IMPLICATIONS FOR OXYGEN ACTIVATION AND CATALYTIC INTERMEDIATES

Martin Moche, John Shanklin, Alokesh Ghoshal and Ylva Lindqvist

J. Biol. Chem. 2003, 278:25072-25080. doi: 10.1074/jbc.M301662200 originally published online April 18, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M301662200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 58 references, 13 of which can be accessed free at http://www.jbc.org/content/278/27/25072.full.html#ref-list-1