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

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Δ9 stearyl-acyl 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 stearyl-ACP desaturase from R. 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 azide 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 carboxylate shift in Δ9 stearyl-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 stearyl-ACP desaturase-azide complex and in the reduced rubrerythrin-azide complex. This suggests that Δ9 stearyl-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 μ-oxo-bridge in oxidized desaturase is bound to the di-iron center as in rubrerythrin and not as reported for the R2 subunit of ribonucleotide reductase and the hydroxylase subunit of methane monooxygenase. The crystal structure of the one-iron depleted desaturase species demonstrates that the affinities for the two iron ions comprising the di-iron center are not equivalent, Fe1 being the higher affinity site and Fe2 being the lower affinity site.

Δ9 stearyl acyl carrier protein desaturase (Δ9 desaturase)1 from R. communis is a soluble enzyme located in the plastid catalyzing the NADPH- and O2-dependent insertion of a cis-double bond between the C9 and C10 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 monooxygenase (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-Glu135 plus α4-Glu143 and -His146 and α2-Glu196 plus α4-Glu229 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. Glu135 is a bidentate ligand to one iron ion (Fe1), and correspondingly, Glu196 is a bidentate ligand to the second iron ion (Fe2). Glu143 and Glu229 both act as bridging ligands of the iron center. Besides these carboxylate ligands, each iron ion has one nitrogen atom ligand, N81 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 ferrie di-iron center needs to be fully oxidized by ferredoxin 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 ferredoxin 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 chain bound 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 hydration, this hypothesis remains to be tested. Modeling stearic acid in the hydrophobic cavity of Δ9 desaturase places the C9-C10 carbon atoms 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 stearyl-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-diferrie 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 oleyl-ACP or of hydrogen peroxide but through an oxidase 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 complexes represent models for the μ-1,2-peroxo intermediate in desaturase catalysis and a model for inhibition by hydrogen peroxide. In addition, we determined the structure of the iron-free apo form of Δ9 desaturase to 3.2-Å resolution and with a single iron ion (Fe1) present in the di-iron center to 2.8-Å resolution. These are compared with crystal structures of other iron-depleted di-iron enzymes, and we discuss the implications for iron insertion in Δ9 desaturase.

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 Scientific, New Brunswick, NJ), in Luria-Bertani broth supplemented with 1% (w/v) glucose to an A660 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. Dissolved oxygen was maintained above 15% and pH was maintained at 6.5. Cell densities at harvest were ~12 A660. 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 Poros 20CM (Applied Biosystems, Foster City, CA). The column was developed with 20 mM HEPES, pH 7.0, 70 mM NaCl. Desaturase-enriched fractions were identified by SDS-PAGE, pooled, and concentrated with the use of an Amicon PM30 ultrafiltrator. The concentrate was subjected to size exclusion chromatography with a TSK G2000SW 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 15–18% 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 in P212121 were as follows: a = 192.9 Å, b = 145.2 Å, and c = 81.8 Å (i.e., ~3% shorter than the original cell due to the use of cryo conditions described above). They contain three dimers in the asymmetric unit. Data were collected at beamline 711 at MAX-LAB in Lund and processed with DENZO (27) and SCALEPACK (27) (Table I).

Crystals of the acetate complex were prepared as described earlier for holoenzyme, except for the addition of 20% (v/v) glycerol to the well solution. This results in a different space group, P3,12, with cell parameters a = b = 94.1 Å and c = 81.7 Å. They contain only one subunit in the asymmetric unit. Data were collected at cryo temperature at beamline X21B at NSLS (Department of Biology, Brookhaven National Laboratory) and processed with MOSFLM (28) and SCALA (29) from the CCP4 suite (30).

The material used for crystallization of the iron-depleted form of desaturase consisted of a 2:2 complex (2 subunits of Δ9 desaturase plus 2 molecules of stearyl-ACP from spinach) verified by electrospray ionization (data not shown). The initial objective was to crystallize the complex between these two protein molecules. The iron depletion was achieved unintentionally by the use of N-2-acetamido-2-iminodiacetic acid buffer, which acted as a chelator. Crystals were obtained at 4 °C with 8–10% (v/v) polyethylene glycol 6000 as the precipitant, 60 mM strontium chloride, 0.1 M N-(2-acetamido)-2-iminodiacetic acid buffer, pH 6.7, and 15% (v/v) glycerol as cryoprotectant. The crystals, which were very pH-sensitive, belong to space group P3,12, with cell axes a = b = 188.0 Å and c = 82.1 Å, and they contain three desaturase dimers in the asymmetric unit. Data were collected at cryo temperature at beamline X25 at NSLS and processed with DENZO (27) and SCALEPACK (27). The crystals with a single iron present in the active site were made at room temperature with pH 4 using 8–12% (v/v) polyethylene glycol 8000, 50 mM potassium dihydrogen phosphate, and 20% (v/v) glycerol as cryoprotectant. The crystals belong to space group P212121 with cell parameters a = 192.4 Å, b = 145.8 Å, and c = 81.9 Å and contain three dimers in the asymmetric unit. Data were collected at beamline 711 at MAX-LAB in Lund and processed with DENZO (27) and SCALEPACK (27).

Structure Determination and Refinement—All structures were solved...
by molecular replacement using the program AMORE (31) with the original dimeric structure of \( \Delta 9 \) desaturase (15) (Protein Data Bank accession number 1AFR) as a search model. Initial refinement of all four structures was performed with the use of the CNS program package (32). This refinement consisted of anisotropic scaling, bulk solvent correction, simulated annealing, conjugate gradient minimization, and isotropic B-factor refinement against the maximum likelihood target. Tight non-crystallographic symmetry restraints were used whenever possible to improve the data to parameter ratio. Averaged maps, calculated by density modification (33), were also utilized for model building carried out in O (34). Final refinement was performed with REFMAC5 (35) using the maximum likelihood residual, anisotropic scaling, bulk solvent correction, and atomic displacement parameter refinement with the “translation, liberation, screw rotation” (TLS) method (36). Each “rodlike” structure most likely represents azide bound to the reduced original structure of the ferrous di-iron center. Therefore, this center with no significant changes in the geometry of the coor-

dinate electron density. One iron-iron distance of 4.1 Å and iron-nitrogen distances of 2.5 Å and 2.6 Å, respectively. To verify the significance of the shift in position of the side chain histidines, and the carboxylates are left in the plane of a dis-

torted octahedron (Figs. 1 and 2). The orientation of the azide ion is clearly defined in the 2\( F_o \) − \( F_c \) electron density map, but some remaining positive electron density is observed in some

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<th>Data collection</th>
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<th>One iron</th>
<th>Acetate complex</th>
<th>Azide complex</th>
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<td>( \text{P}2_{12}2_{12} )</td>
<td>( \text{P}3_12 )</td>
<td>( \text{P}2_{12}2_{12} )</td>
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<td>2.4 (2.46-2.4)</td>
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<td>0.058 (0.252)</td>
<td>0.043 (0.283)</td>
<td>0.057 (0.299)</td>
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\[ R_{sym} = \frac{\sum_{hkl} F(hkl) \left| F(hkl) \right|}{\sum_{hkl} F^2(hkl)} \]
\[ R-factor = \frac{\sum_{hkl} \left| F(hkl) \right| - k \sum_{hkl} F^2(hkl)}{\sum_{hkl} F^2(hkl)} \]

**RESULTS**

**Crystal Packing and Space Groups**—Crystals from three space groups, \( \text{P}2_{12}2_{12} \), \( \text{P}3_12 \), and \( \text{P}3_1 \), with approximately the same length of their shortest cell axis have been obtained during this work (Table I). As the desaturation dimers have a strong tendency to pack along a 3-fold screw axis, being noncrystallographic in the \( \text{P}2_{12}2_{12} \) 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 \( \Delta 9 \) desaturase determined at room temperature (Table I). Minor differences are localized to three areas where all of the structures show very high B-factors and in some cases 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 \( \text{P}3_12 \), where they appear disordered. In regions of badly defined electron density, side chain occupancies have been set to zero.

For the azide complex in \( \text{P}2_{12}2_{12} \) and the acetate complex in \( \text{P}3_12 \), 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 Glu229 has more flexibility than other iron center ligands, as evidenced by higher B-factor and less well defined electron density.

**Azide Binding to the \( \Delta 9 \) 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 center in a \( \mu \)-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 2\( F_o \) − \( F_c \) 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-P212121 and the acetate-P3112 structure.

**Acetate Binding to the Δ9 Desaturase Di-iron Center**—The overall structure of desaturase is very similar between this P3112 structure and the P212121 original structure (15). The root mean square deviation of 0.50 Å for 337 Cα-carbon atoms (Table II) is only slightly higher than for the P212121 azide complex, 0.36 Å over 345 Cα-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), Glu196, 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 Glu196 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 Glu196 is therefore considered a valid feature of the structure. The orientation of Glu196 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 Δ9 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 unchanged 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 or 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 Δ9 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 Δ9 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 values, 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 2Fo − Fc 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. 5b). 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. 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 Δ9 desaturase dimer (Fig. 5a). The distance from the high affinity iron (Fe1) sites and the ion 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 Δ9 desaturase, the μ-oxo bridge is either protonated or lost (41). Spectroscopy further predicted two different, pH-dependent, binding modes of azide (μ-1,3-bridging and μ1-terminal), with ~90% 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 μ-1,3 binding mode but also shows some difference density, suggesting the presence of a small proportion of azide in a μ-1,1 binding mode. In fact, the spectroscopic data for the second conformation of the ferric azide complex could be interpreted as either μ1-terminal with a protonated μ-oxo bridge or as μ-1,1 coordination, where the μ-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 μ-1,1-bridging in both R2 mutants occupying the position of the μ-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 ruberythrin (Fig. 7), showing virtually identical coordination (44). As in desaturase, binding of azide to the reduced state of ruberythrin introduces no carboxylate shifts in the surrounding iron ligands, and it binds azide in the same fashion.

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An important difference in the structure of the oxidized iron center in rubrerythrin and in oxidized R2 and MMOH is the position of the $\mu$-(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 $\mu$-oxo-bridge of azide is known to displace the $\mu$-oxo-bridge in desaturase, and we thus suggest that the $\mu$-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 $\Delta 9$ desaturase crystal structure.

In a study of magnetic circular dichroism in chemically reduced $\Delta 9$ 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 P3112 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 $\Delta 9$ desaturase, we suggest that acetate has replaced the $\mu$-oxo bridge, but in MMOH it has replaced a second solvent bridge. In both $\Delta 9$ 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 $\Delta 9$ desaturase. Both in 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 $\Delta 9$ desaturase. The difference is that the histidine ligand of the low affinity Fe2 forms a hydrogen bond to a conserved glutamate (Glu142) in $\Delta 9$ desaturase and a
The conserved Glu97 of rubrerythrin bound with water instead of oleoyl-ACP can be prepared (21, 22). The azide peroxide intermediate species that decays with formation of water (22) is required for catalysis and reactivity of the di-iron clusters. The importance of correct positioning of the histidines for the geometry and reactivity of the di-iron clusters 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 PeroxoFerric 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 that follows 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, raise the possibility that the stable 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 peroxoFerric 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 Glu97 of rubrerythrin, catalyzing the required proton transfer (44, 52), is occupied by Thr199 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).

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 pKₐ 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.

Fig. 7. Azide complexes of desaturase (A) and rubrerythrin (B) viewed in equivalent orientations. Note the almost identical rotamer conformation between the iron ligands in desaturase and rubrerythrin. Sequence-conserved Thr199 of desaturase corresponds to the conserved Glu97 of rubrerythrin.

Fig. 8. Comparison of acetate complexes of desaturase (A) and methane monoxygenase hydroxylase (IMMO) (B). Hydrogen bonds to iron ligands are indicated with dotted lines. Glu142 and Asp228 in desaturase, corresponding to Asp143 and Asp242 in MMOH, make hydrogen bonds to the histidine iron ligands.
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 as active for intake of iron into desaturases; indeed, in overexpression of the recombinant enzyme, iron insertion occurs spontaneously. The presence of an auxiliary binding site for metal ions at the subunit surface, as observed with a bound stromolion 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 occur at this site, and the iron ions would then be transported along the water channel connecting this site to the iron center.

Conclusions—The Δ9 desaturase active site 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 catalytically competent species present during productive turnover. The strong structural similarity to the reduced rubrerythrin 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 in rubrerythrin 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.

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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

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