Controlling Electron Transfer in Acyl-CoA Oxidases and Dehydrogenases

A STRUCTURAL VIEW*

Plants produce a unique peroxisomal short chain-specific acyl-CoA oxidase (ACX4) for β-oxidation of lipids. The short chain-specific oxidase has little resemblance to other peroxisomal acyl-CoA oxidases but has an ~30% sequence identity to mitochondrial acyl-CoA dehydrogenases. Two biochemical features have been linked to structural properties by comparing the structures of short chain-specific Arabidopsis thaliana ACX4 with and without a substrate analogue bound in the active site to known acyl-CoA oxidases and dehydrogenase structures: (i) a solvent-accessible acyl binding pocket is not required for oxygen reactivity, and (ii) the oligomeric state plays a role in substrate pocket architecture but is not linked to oxygen reactivity. The structures indicate that the acyl-CoA oxidases may encapsulate the electrons for transfer to molecular oxygen by blocking the dehydrogenase substrate interaction site with structural extensions. A small binding pocket observed adjoining the flavin adenine dinucleotide N5 and C4a atoms could increase the number of productive encounters between flavin adenine dinucleotide (FAD) as a cofactor. The cofactor gets reduced to FADH in the first half-reaction concomitant with acyl-CoA oxidation. FADH is reoxidized by molecular oxygen in the second step, thereby generating \( \text{H}_2\text{O}_2 \), an intracellular signaling molecule.

Unlike plants, two parallel and distinct β-oxidation pathways exist in mammals, the peroxisomal β-oxidation pathway and a mitochondrial β-oxidation pathway. In mitochondrial β-oxidation, the first step is catalyzed by the acyl-CoA dehydrogenase family (ACD) (1), which is related to ACXs. The FADH in ACDs is not, however, reoxidized by molecular oxygen but rather by another flavoprotein, the electron transfer flavoprotein, which transfers electrons to the electron transport chain. As a result, the oxidation of fatty acids/amino acids and the generation of ATP molecules are linked in mitochondrial β-oxidation (2). It is puzzling that natural selection has not forced plants to utilize the mitochondrial electron transport chain for general lipid oxidation despite the apparent ATP advantage of this pathway. This might reflect less stress on the ATP requirement and a higher demand for lipid turnover and excess oxygen management in plants.

Six genes for ACX isozymes have been identified in Arabidopsis thaliana. Five encode for proteins of ~75 kDa (3–5). The proteins have different but overlapping substrate chain length specificity (3) and one, ACX1, has been linked to the synthesis of the plant hormone jasmonate (6, 7). The majority of the characterized enzymes in the ACX family form dimers in solution and have an extra C-terminal domain (3, 8–10) that is not found in short, medium, and long chain-specific ACDs. The extra C-terminal domain resembles the C-terminal domain observed in very long chain-specific ACDs, which also form dimers (11, 12). The last A. thaliana ACX gene encodes for a short chain-specific enzyme (ACX4). It is a 50-kDa enzyme with little sequence identity to the other ACXs, yet it still catalyzes the same reoxidation step with the transfer of electrons from FADH to molecular oxygen (13).

In mammals, the peroxisomal β-oxidation does not go to completion. Instead short chain fatty acids are transferred to the mitochondrial β-oxidation cycle. The peroxisomal short chain-specific ACX is unique to plants (14) and seems to be an evolutionary link between the two alternative β-oxidation systems. The very long chain-specific ACD could have evolved in a similar way to the very long chain-specific ACD. Short chain-specific ACX shares the functional, homotetrameric state of short, medium, and long chain-specific ACDs (15) and has a higher sequence identity to mitochondrial dehydrogenases.
ACX4 Expression, Purification, and Crystallization—The A. thaliana gene encoding non-His-tagged ACX4 was amplified by PCR using a cDNA clone (GenBank® accession No. T46525) obtained from the Arabidopsis Biological Resource Center as a template. The primers used were 5′-CTATCGCCGATATGGCGGTGCTTTCATCT-3′ and 5′-GTCATCTCGAGTCAGAGACGGCTACGTGTACGCGG-3′. The amplified DNA was inserted into the pET24 vector (Novagen) using Ndel and XhoI restriction sites. The resulting plasmid was transformed into E. coli BL21(DE3), and the cells were grown in LB medium containing 50 mg/liter kanamycin at 37 °C. Protein expression was induced at an A600 of 0.6 – 0.8 by the addition of isopropyl 1-thio-β-D-galactopyranoside to a final concentration of 0.5 mM. The cells were harvested by centrifugation 3 h after induction. The harvested cells were resuspended in 10 ml of BugBuster (Novagen) per liter of cell culture, with the addition of 0.5 ml of lysozyme (25 mg/ml; Sigma) per 10 ml of BugBuster. After cell lysis, benzonase (Sigma) was added at 1 μl/10 ml of BugBuster. The cell debris was removed by centrifugation at 20,000 × g for 60 min. The protein solution was dialyzed against 30% (w/v) polyethylene glycol 8000 to reduce the volume to ~8 ml. The concentrated sample was applied onto a size exclusion column (Superdex75, GE Healthcare) using 20 mM HEPES buffer, pH 7.0, 100 mM NaCl, and 10 μM FAD as a running buffer. Fractions containing enzyme were pooled and applied directly onto an anion exchange column (MonoQ; Pharmacia Corporation) equilibrated with 20 mM HEPES buffer, pH 7.0, containing 100 mM NaCl and 10 μM FAD. Highly purified ACX4 (95%) was collected in the flow through. The protein was dialyzed against 20 mM HEPES buffer, pH 7.0, and 10 μM FAD and concentrated to 10 mg/ml in a Centriprep YM-10 filter unit (Millipore). After flash freezing in liquid nitrogen, the protein was stored at −20 °C. Crystals were obtained by the hanging drop vapor diffusion technique mixing equal volumes (2 μl) of ACX4 (10 mg/ml in 20 mM HEPES buffer, pH 7.0, 10 μM FAD) and precipitating solution (10–14% (w/v) polyethylene glycol 8000, 100 mM cacodylate, pH 6.5) with the addition of 0.5 μl of 20% (v/v) benzamidine-HCl and 0.5 μl of 10 mM AcAcCoA and equilibrating against precipitating solution (1000 μl) at 22 °C. The crystals developed over 4 days. Cryoprotection was achieved by transfer of the crystals to 25% (v/v) glycerol by gradually increasing the concentration by 5%. Crystallization conditions for His-tagged ACX4 without AcAcCoA have been reported previously (16). The use of the non-His-tagged construct and inclusion of AcAcCoA in the crystallization conditions dramatically improved the reproducibility and quality of the ACX4 crystals.

EXPERIMENTAL PROCEDURES

Data Collection and Structure Determination—Diffraction data from the ACX4-AcAcCoA complex were collected at the I1711 beam line, MAX-lab, Lund University, Sweden. The crystal form was non-isomorphous with the crystal form observed with the His-tagged enzyme (16). ACX4-AcAcCoA crystals belong to space group P321, a = b = 144.7 Å, c = 149.2 Å, contain four molecules/asymmetric unit and have a Matthews coefficient of 2.4 Å³/Da and a solvent content of 47%. The best ACX4-AcAcCoA crystals diffracted to 2.7 Å, whereas the previously characterized crystals of His-tagged ACX4 diffracted to 3.9 Å. Diffraction data were indexed and integrated with MOSFLM (17) and scaled with SCALA (18). The data collection statistics are given in Table 1.

The structure of ACX4-AcAcCoA was solved by the molecular replacement technique as implemented in the program Phaser (19) included in the CCP4 program package (20) with a tetramer of rat short chain acyl-CoA dehydrogenase (21) as the search template. The structure of the His-tagged ACX4 was solved by molecular replacement using MOLREP (22) using one subunit of the refined ACX4-AcAcCoA structure as model. The crystal had six molecules in the asymmetric unit. The ACX4-AcAcCoA structure was refined using iterative cycles of crystallography NMR software-simulated annealing refinement against the maximum likelihood target (23) or REFMAC5 (24) refinement alternating with manual rebuilding of the molecular structure using the O graphics software (25). After resetting Biso values to 20, a last round of REFMAC5 refinement, including translation/libration/screw (TLS) refinement, was applied. This was followed by restrained positional refinement and supplemental Biso refinement (26). Each of the four subunits defined a TLS group. Of the 439 residues in each subunit in the ACX4-AcAcCoA structure, all were comprised in the model, except 16 residues in the N terminus and 8 residues in the C terminus (including the PTS1 signal sequence). The occupancy of the AcAcCoA ligand was refined.

Because of the low resolution of the His-tagged ACX4 data, only one round of crystallography NMR software-simulated annealing refinement was conducted, applying strict NCS between the six subunits in the asymmetric unit and not refining the B-factors nor including solvent molecules. The simulated annealing was followed by 10 cycles of TLS refinement in REFMAC5, first setting Biso = 20 for all protein atoms and applying one TLS group for each protein chain. Tight NCS restraints were used in the subsequent restrained positional refinement followed by refinement of the residual Biso. This procedure resulted in a drop in Rfree from 28 to 25%. In His-tagged ACX4, the His tag and N- and C-terminal residues were disordered. The final Rfree and Rfree values of the ACX4-AcAcCoA structure were 19 and 23%, respectively, whereas the values for His-tagged ACX4 were 25 and 25%. The relatively low R-factor for His-tagged ACX4 at 3.9 Å resolution is probably a consequence of the use of the higher resolution
ACX4-AcAcCoA structure as a starting point for the refinement. Refinement statistics are found in Table 1. Ramachandran plots of both structures have 92% of the residues in the most favored regions and one residue, Leu-393, in the γ-turn region. The coordinates and structure factors have been deposited in the Protein Data Bank (PDB) with the accession numbers 2IX5 and 2IX6, respectively.

**Structural Analysis**—Structural classification of the ACX4 structure took advantage of the SCOP data base (27). The search for structural similarity to the N-terminal ACX4 extension was performed using the Macromolecular Structure data base secondary structure-matching facility against structures deposited in the PDB (28). Protein superposition was achieved with Lsqman (29), and protein-protein interactions were analyzed via the protein-protein interaction server (30). Helanal (31), a program for characterization of helix geometry, was applied to analyze helix curvature, whereas secondary structure prediction for very long chain-specific acyl-CoA dehydrogenase (VLCAD) was done using Jpred (32). The program Surfnets was used for binding pocket analysis (33). In this analysis, a CoA molecule was left in the pocket to block the entrance, and the volume of the remaining cavity was calculated. Electrostatic potentials were calculated using the full-charge force field and the program Delphi (Accelrys), setting the ionic strength of the solvent to 0.1 M and keeping default values for the other parameters. A similar result was obtained by calculating the electrostatic potentials using the Amber94 force field in the program MOE (Chemical Computing Group).

**RESULTS**

**Three-dimensional Structure**—ACX4 was co-crystallized with the FAD cofactor and a substrate analogue, acetoacetyl-CoA (AcAcCoA). The ACX4 subunit adopted the canonical dehydrogenase fold with an N-terminal α-helical domain (residue 57–167), followed by a middle β-strand domain (residue 168–276) and a C-terminal four-helix bundle (residue 277–431) (Fig. 1A). The β-strand domain belongs to the acyl-CoA dehydrogenase NM domain-like superfamily and the C-termi-
The role of the N-and C-terminal tails of ACX4 in burying the FAD cofactor. The N terminus of subunit B (green) and the C terminus of subunit C (tan) are made transparent to show how they enclose the FAD cofactor.
acquisition code 1SIR) (15) has a high sequence identity to ACX4 (33%) as well as a high structural resemblance (root mean square deviation of 1.6 Å over 98% of the Cα atoms). Human GCD has a cleavable 44-amino-acid-long N-terminal mitochondrial targeting sequence with no significant homology to the N-terminal sequence of ACX4 (43). The N-terminal extension is found only in a few of the bacterial GCDs that share high sequence identity with ACX4.

The Substrate Binding Pocket—The substrate analogue AcAcCoA is bound with the thioester bond sandwiched between the catalytic base and the FAD isoalloxazine ring in a cavity between the β-strand domain and the C-terminal four-helix bundle (Fig. 4). The ACX4:AcAcCoA crystals have a solvent content of 47%, and solvent channels surround the tetramer in the crystal packing. Only the D sub-

FIGURE 3. Superposition of A. thaliana ACX1 (green and gray) and ACX4 (beige and black) dimer. The N-terminal extension of ACX4 (red) covering the electron transfer flavoproteins docking area of ACDs is superposable on the C terminus of ACX1 (orange).

FIGURE 4. Acyl-CoA binding pocket of ACX4. A, stereo figure of ACX4 substrate binding pocket of the AcAcCoA complex with the σa-weighted 2mF – ΔF electron density map shown at a 1σ level. The AcAc moiety has no observable electron density and has not been included in the model. B, docking of octanoyl-CoA in ACX4. A rotamer change (turquoise) of Leu-137 can widen the pocket and allow the binding of acyl-CoA substrates up to a length of eight carbon atoms. C, superposition of ACX4 helix G with (beige) and without (green) AcAcCoA bound. The helix has moved ~2 Å toward the substrate analogue. Side chains of the shifted part in helix G making interactions with AcAcCoA are shown in sticks, hydrogen bonds are represented by black dashed lines, and hydrophobic interactions by red lines.
unit has restricted access to the substrate binding pocket due to crystal packing. This could be the reason why the ligand in the D subunit has higher occupancy and lower B-factors (Table 1) than the ligands in the A and C subunits, as ligand might have escaped the active site during cryoprotection. Only the CoA part of the AcCoA inhibitor co-crystallized with the protein has significant electron density in the structure (Fig. 4\(^a\)). The binding of AcAcCoA to ACX4 results in a shift in helix G of \(\sim 2\) Å (Fig. 4C). Helix G is a 10-turn \(\alpha\)-helix with a 16° curvature in the native ACX4 structure. The helix straightens upon substrate binding, and the curvature diminishes to 10°.

The residues lining the acyl binding pocket are all hydrophobic, except for Thr-134, Gln-294, and the catalytic Glu-408. The acyl binding pocket is defined by residues Thr-134, Ala-183, Phe-428, Phe-316, and Val-417 and between the acyl-CoA substrates nucleotide moiety and the CoA adenine ring forms hydrophobic interactions to Arg-420, and hydrophobic interactions can also be seen between pantothenate and Phe-277, Ile-413, Leu-174, Ala-183, Phe-428, Phe-316, and Val-417 and between the \(\beta\)-mercaptoethyamine part and Trp-172 and Glu-408 (Fig. 4A). The binding of AcAcCoA to ACX4 results in a shift in helix G of \(\sim 2\) Å (Fig. 4C). Helix G is a 10-turn \(\alpha\)-helix with a 16° curvature in the native ACX4 structure. The helix straightens upon substrate binding, and the curvature diminishes to 10°.

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stabilizing interactions to the CoA phosphates are not generally observed in ACD-substrate complexes (15, 21, 37, 46), and although an arginine residue corresponding to Arg-420 is found in many structures, no \( \pi-\pi \) interaction is observed. In ACD-substrate complexes, the adenine ring interacts with residues from the N terminus of the G-helix. Consequently, the substrate binding pocket and the FAD edge are at least as restricted to solvent access in ACX4 as in dehydrogenases.

No well ordered water molecules are observed in the ACX4-AcAcCoA binding pocket, and 45 Å\(^3\) of the pocket are apparently unoccupied. In SCAD and MCAD, well ordered water molecules can be seen in the part of the pocket not occupied by the acyl-CoA substrates (21, 37).

**Substrate Specificity and Oligomeric State**—Previous studies show that ACX4 as well as rat SCAD have maximal activity for butyryl-CoA and only weak activity for substrates larger than octanoyl-CoA (13, 47). Placing a C\(_8\) chain in the acyl binding pocket as defined in the AcAcCoA complex shows that the pocket is too restricted to accommodate this substrate. Studies of human medium chain-specific acyl-CoA dehydrogenase (MCAD) with and without substrate show that residue Glu-99 at the end of the pocket is flexible and changes rotamer for the pocket to accommodate the octanoyl-CoA substrate (37). The residue corresponding to the MCAD Glu-99 residue in ACX4 is Leu-137. A reorientation of this residue to one of its other preferred rotamers could make room for octanoyl-CoA binding (Fig. 3B), whereas longer substrates cannot be accommodated.

Compared with ACX1, the ACX4 pocket is shorter and narrower. From the overall superposition, this effect can be said to be caused by differences in the relative positions of helices E, D, and G. This makes the acyl binding pocket in ACX4 solvent-accessible from the CoA-binding site only, whereas ACX1 has a channel-like pocket, which is solvent-accessible from both ends (10). When substrate is bound in the pocket, the CoA plugs the entrance and the pocket becomes solvent-inaccessible. In this respect, ACX4 is more similar to ACDs than to ACX1. The closed ACX4 binding pocket does not comply with the proposed role of the open channel in ACX1, favoring the reoxidation of FADH\(^+\) by molecular oxygen and allowing fast exit of the H\(_2\)O\(_2\) formed (10).

ACX1 and rat acetyl-CoA oxidase II form dimers and have an additional C-terminal \( \alpha \)-domain compared with ACX4 (Fig. 3). The same is the case for VLCAD (48, 49). The extra C-terminal helical domain in ACX1 contributes to FAD binding and might be necessary to stabilize the structural integrity of the individual subunits when the huge internal cavity is present. VLCADs must be expected to adopt an open acyl binding pocket as observed in ACX1 (10) and rat acetyl-CoA oxidase (9). The extra domain of ACX1 might result from an early domain duplication event, because the structural elements in the two C-terminal domains are the same. The sequence identity between the two domains is negligible, however, and only three of the four helices in the bundles (helices G, H, and I versus helices M, O, and P) can be superposed. The domain has equally low sequence identity to the C-terminal domain of SCAD, to the ACX4 C-terminal domain, and to the extra C-terminal domain found in VLCADs that can be predicted to have a similar helical topology. Short chain-specific oxidases and SCADs have no need for the huge substrate binding pore and therefore no need for an extra C-terminal domain for stabilization of FAD binding.

To accommodate long substrates and form the very wide pocket that is present in ACX1, a large shift of helices D and G is necessary. This shift, which could be an effect of a missing helix B and a short \( \alpha-C-\alpha \)-loop in ACX1, breaks all contacts between helices D and G. In ACX4 and in dehydrogenases, helices D and G make extensive interactions and link the N- and the C-terminal four-helix bundles.

**Oxidase Requirements**—Despite the fact that the structure of ACX4 very much resembles the ACD family, ACX4 is an oxidase and has been shown not to harbor any ACD activity (13). A hypothesis put forward by Wang and Thorpe (50) suggests that the low oxygen reactivity of the dehydrogenase family is a result of efficient desolvation of the active site, thereby suppressing the formation of the incipient superoxide anion radical. When the two very similar structures of ACX4 and human GCD are compared, the amino acids lining their FAD binding pockets are very similar. Of 20 amino acids defining the thioester pocket and the pocket around the FAD isoalloxazine ring, 13 amino acids are identical, four are chemically similar, and only two strikingly different (ACX4 residues Gly-207 and Asn-255) (Fig. 5). Gly-207 corresponds to Thr-170, whereas Asn-255 corresponds to Thr-217 in human GCD and both environ the FAD N5 atom (Fig. 5). Gly-207 and Asn-255 are conserved throughout the ACX family. The Asn-255 residue is located on \( \beta \)-strand 7 and is making interaction with the backbone carbonyl group of conserved Trp-205, which is an important residue for positioning the FAD isoalloxazine ring (Fig. 5). When comparing the substrate binding cavities of the two proteins, a distinct unoccupied volume is observed adjacent to the FAD N5 and C4a atoms in the ACX4 structure, a position occupied by Thr-170 in the GCD structure (Fig. 5). This superfluous volume in the ACX FAD binding pocket could ensure molecular oxygen accessibility to FAD and focus encounters between molecular oxygen and FAD at the FAD C4a atom, although the solvation of the active site seems at least as restricted as in the dehydrogenase family. The position of the pocket does not bear any resemblance to the position of the postulated peroxide molecule in the high resolution structure of d-amino acid oxidase (51), nor does it provide a straightforward route for proton transfer to the emerging peroxide. However, Asn-255 might provide a hydrogen bond to stabilize the formation of the superoxide anion intermediate (52) in this position, a hypothesis that can be tested by mutagenesis experiments. The ACXs lack the hydrogen bond found between the ACD active site threonine and FAD N5, which in ACDs will contribute to charge delocalization and, hence, lower oxygen reactivity. Proteolytic cleavage of bovine milk xanthine dehydrogenase/xanthine oxidase converts the enzyme from a dehydrogenase to an oxidase. The conversion results in a dramatic change in the electrostatic potential of the flavin binding pocket toward a more electronegative environment (41). A similar difference in the electrostatic potential in the FAD binding pocket is not seen in a comparison of ACX4 and human GCD (Fig. 5).
Conclusion—The suggestion from earlier structures of acyl-CoA oxidases that the wider substrate channel could account for higher oxygen reactivity does not hold for ACX4. The structural similarity between ACX4 and the ACD family is striking, and there is no obvious difference in oxygen accessibility in the two enzyme families. An analysis of the substrate binding pocket shows that the ACX4 pocket is smaller rather than larger than the pocket in the ACD family and that the substrate pocket in the tetrameric enzymes in both families is blocked from solvent access when substrate is bound.

ACX4 shares a tetrameric state with the ACD family. It appears that the function of the extra C-terminal domain, present in dimer-forming oxidases such as ACX1 and preventing the formation of a tetramer, is to stabilize a large acyl binding pocket. The oligomerization state is not correlated with oxygen reactivity.

The ACX4 N-terminal tail and the superposable ACX1 C-terminal tail may be essential in blocking the path from the FAD isosalloaxazine ring to the solvent. The present structural study suggests that uncontrolled electron transfer is effectively stopped by the terminal loops in this part of the enzymes and that the combined action of this blockage and the extra volume around the FAD N5 and C4a atoms, which focus the O₂ encounters to this region, make ACX4 an oxidase rather than a dehydrogenase.

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