Acyl-CoA dehydrogenases and acyl-CoA oxidases
Structural basis for mechanistic similarities and differences

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Acyl-CoA dehydrogenases and acyl-CoA oxidases are two closely related FAD-containing enzyme families that are present in mitochondria and peroxisomes, respectively. They catalyze the dehydrogenation of acyl-CoA thioesters to the corresponding trans-2-enoyl-CoA. This review examines the structure of medium chain acyl-CoA dehydrogenase, as a representative of the dehydrogenase family, with respect to the catalytic mechanism and its broad chain length specificity. Comparing the structures of four other acyl-CoA dehydrogenases provides further insights into the structural basis for the substrate specificity of each of these enzymes. In addition, the structure of peroxisomal acyl-CoA oxidase II from rat liver is compared to that of medium chain acyl-CoA dehydrogenase, and the structural basis for their different oxidative half reactions is discussed.

Introduction
There are nine known members in the acyl-CoA dehydrogenase (ACAD) family, including the recently identified ACAD-9, which is homologous to the very long chain acyl-CoA dehydrogenase [1]. Five members of the ACAD family are involved in fatty acid β-oxidation; these are short, medium, long and very long chain acyl-CoA dehydrogenase (SCAD, MCAD, LCAD and VLCAD1, respectively), and ACAD-9 (hereafter referred to as VLCAD2). The four other members are involved in amino acid oxidation pathways; they are iso(3)valeryl-CoA dehydrogenase (i3VD) for leucine, iso(2)valeryl-CoA dehydrogenase (i2VD, also known as short/branched chain acyl-CoA dehydrogenase or 2-methylbutyryl-CoA dehydrogenase) for isoleucine, isobutyryl-CoA dehydrogenase (iBD) for valine, and glutaryl-CoA dehydrogenase (GD) for lysine and tryptophan. With the exception of VLCADs, all of these are soluble homotetramers with a subunit mass of approximately 43 kDa, with each subunit containing one FAD.

Like VLCAD1, ACO is a homodimer with a subunit mass of 73 kDa with one FAD per monomer and is bound to the matrix side of the mitochondrial inner membrane [2]. Three-dimensional structures of rat SCAD [3], pig and human MCAD [4,5], i3VD [6], and GD [7] have been solved. As MCAD has been studied the most extensively, both biochemically and structurally, this review discusses the MCAD structure in detail as the prototype. For the other ACAD family members, only those features that differ from the MCAD structure will be discussed. The structure of a peroxisomal acyl-CoA oxidase (ACO) was recently determined [8] and is compared to MCAD. These structures are compared with respect to the structural basis for their different oxidative half reactions. ACO is reoxidized by molecular oxygen (i.e. a true oxidase), whereas MCAD is reoxidized by transferring electrons to another flavoprotein, electron transfer flavoprotein (ETF). However, it should be noted that, in its ligand-free form, the reduced flavin of MCAD has relatively high oxygen reactivity compared to the product-bound form, which has virtually no oxygen reactivity [9–11].

Amino acid sequences and quaternary structure
The amino acid sequences of eight members of the ACAD family, from several different sources, have been deduced from their cDNA sequences [12,13]. With the exception of VLCAD, the sequence identities among these members range from 35–45% and are evenly distributed over the entire polypeptide span, strongly suggesting that these enzymes originated from a common ancestral gene. Figure 1 shows a structure-based sequence alignment of the ACADs and ACO whose three-dimensional structures have been determined. A common evolutionary path is also suggested by the similarity in their overall three-dimensional structures.

Like VLCAD, ACO is a homodimer with a subunit molecular mass of 75 kDa. The sequence identities of the N-terminal approximately 400 residues of VLCAD and...
Fig. 1. Structure-based sequence alignment of pig MCAD, rat SCAD, bacterial SCAD from M. elsdenii, human i3VD and rat ACO-II. α-Helices are indicated with cylinders and, β-strands with arrows. Residues that are identical in all four ACADs or all five enzymes including ACO-II are shown in a light blue box and those that are similar are shown in a pink box. Residues that line the binding cavity for the acyl chain moiety of the thioester substrate are in red. As the structure of ACO-II is that of the uncomplexed enzyme, its cavity lining residues are not known and therefore not marked. Catalytic glutamates are marked with a white letter and red background.
ACO to the MCAD sequence (all human enzymes) are 36% and 31%, respectively; however, the remaining C-terminal approximately 180 residues of each, which are not present in other ACADs, do not share any significant similarity to each other or to any other known proteins in the database. These facts indicate that VLCAD and ACO share a common ancestral gene with ACADs, but have diverged and fused with another gene to bind to the mitochondrial membrane (VLCAD) or to become an oxidase in peroxisomes (ACO).

In addition, recently a large number of ACAD homologs have been identified both in eukaryotes and prokaryotes; and the numbers are likely to increase as more sequence data becomes available. Their functions range from steroid side chain cleavage [14] to antibiotic synthesis [15] and to stress response [16,17].

The three-dimensional structure of MCAD

The structures of MCAD have been solved in binary complexes with substrates or inhibitors having various fatty-acyl chain lengths, as well as in the uncomplexed form [4,18]. The overall polypeptide fold of a monomer of MCAD is shown in Fig. 2A. The monomer is composed of three structural domains of approximately equal size: the

![Fig. 2. Ribbon diagrams of the MCAD structure.](image-url)
N-terminal and C-terminal domains consist mainly of α-helices and are packed together in three-dimensions, forming the core of the tetrameric molecule, whereas the middle domain is composed of two orthogonal β-sheets and lies at the surface of the molecule (Fig. 2B). The tetrameric molecule is a dimer of dimers in a tetrahedral arrangement with an overall diameter of about 90 Å (Fig. 2B). The interactions between the two monomers in the dimer are extensive, involving the FAD binding site, whereas those between the two dimers are mainly of helix–helix interactions, similar to the ones seen in a four-helix bundle structure. The bound FAD has an extended conformation with the isoalloxazine ring located at the crevice between the two α-helix domains and the β-sheet domain within one monomer, and its adenosine moiety lies at the interface between two monomers (Fig. 2). The fatty-acyl portion of the thioester substrate is bound at the re-face of the flavin ring buried inside the monomer between helices G and E and the loop between J and K (Fig. 2A). The cavity is deep enough to accommodate substrate with an acyl chain length of up to 12 carbons. In addition, the base of the cavity is wide and ‘upside down Y’ in shape so that it can accommodate the α-end of the acyl chain of C12-CoA in two different conformations, as seen in the structure of human MCAD in complex with C12-CoA [5]. Figure 3 shows the surface that lines the substrate-binding cavity of MCAD. The middle of the binding cavity is long and narrow, just wide enough to accommodate the extended pantetheine chain of the substrate. On the other hand, the adenosine-3'-phosphate-5'-diphosphate portion of the CoA moiety is partially exposed to solvent at the interface of the two monomers (Fig. 3). This funnel shaped crevice probably serves as the entrance to the binding cavity. The C2-C3 bond of the substrate is sandwiched between the carboxyl group of Glu376 (catalytic base) and the isoalloxazine ring of FAD, perfectly poised for the α-β dehydrogenation reaction (Figs 3 and 4). The carbonyl oxygen of the thioester substrate is hydrogen bonded to both the 2'-OH of the ribityl chain of FAD and the amide nitrogen of Glu376 [4]. These interactions are important in the precise positioning and alignment of the flavin, substrate, and Glu376 for optimal catalysis. In addition, they are responsible for the acidification of the α-proton of the substrate.

No major changes in tertiary and quaternary structures have been observed upon binding the substrate; however, there are many subtle but significant changes in side chain conformation of the residues that line the active site cavity. The most pronounced changes are observed in Glu376 (catalytic residue), Tyr375, and Glu99. The carboxylate of Glu376 moves toward the C2 atom of the bound substrate poised to abstract the C2 proton. The side chains of Tyr375 and Glu99 adopt different conformations to accommodate the substrate. In the absence of bound substrate, the active site cavity is occupied by a well-ordered string of water molecules that are successively displaced as the length of the fatty acyl chain increases until C12-CoA binds, at which point all the water molecules are expelled [19]. This is probably how MCAD can accommodate substrates with a broad range of fatty acyl chain lengths with C8- and C10-CoA at the highest rate [9,20]. For the shorter chain substrates (e.g. C4-CoA or shorter), it must be entropically unfavorable to have several water molecules in the active site cavity, while the entrance of the cavity is blocked with the C4-CoA.

Structures of other acyl-CoA dehydrogenases

Four other ACAD structures have been determined: rat SCAD [3], a bacterial SCAD [21], human i3VD [6] and human GD [7]. As expected from the primary sequence similarities, the overall polypeptide folds of these other ACADs are very similar to that of MCAD. The root-mean-square deviations between Cα atoms of MCAD and those of the other four ACAD structures that have been

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**Fig. 3. A stereo view of the active site cavity of MCAD.** The cavity surface is shown with a green transparent surface. The bound substrate, dodecanoyl-CoA, is shown with ball-and-sticks, with the last four carbon atoms (smaller balls) of the substrate modeled in two different conformations. Residues lining the cavity are shown with stick models. Atoms in the residues are colored as follows: carbon, black; nitrogen, blue; oxygen, red; sulfur, green; and phosphorus, purple. The substrate is within the binding cavity, which is 'outside' the molecular surface, and the lining residues are 'inside' the molecular surface. The adenosine pyrophosphate moiety is partially exposed to solvent and the pantetheine-fatty acyl portion of the substrate is completely buried inside the molecule. The molecular surface was generated with a 1.4 Å radius probe using the program GRASP [40].
determined to date range from 1.2 Å to 1.7 Å, excluding the N- and C-termini and the loop regions where a few insertions and deletions occur (Fig. 1). Furthermore, the mode of substrate binding is also conserved, including the two hydrogen bond interactions from the carbonyl of the substrate thioester to the 2¢OH of the ribityl chain of the FAD and to the main chain amide nitrogen of the residue corresponding to Glu376 of MCAD. Therefore, in this section, only features unique to each of these other ACAD structures will be discussed.

Short chain acyl-CoA dehydrogenase

Structures of both rat SCAD (rSCAD) and a bacterial SCAD from Megasphaera elsdenii (bSCAD) have been determined [3,21]. As expected, the catalytic residues are Glu368 in the rat enzyme and Glu367 in the bacterial enzyme, both of which are homologs of Glu376 in MCAD (Fig. 1). The binding cavities of rSCAD and bSCAD for the fatty acyl moiety of the thioester substrate are shown in Fig. 5A and 5B, respectively, and they are very similar in size and shape. They are shallower than the cavity of MCAD, which is consistent with the shorter chain substrate specificity of SCADs. Two features contribute to the shallow binding cavities in SCADs: in SCADs, as well as in i3VD and GD, all of which are specific for shorter substrates than MCAD, there is one extra residue inserted in the middle of helix E (Asn96 in rSCAD), compared to helix E in MCAD (Fig. 1). This insertion causes a bulge in helix E and brings it closer to helix G, making the binding cavity shallower. In addition, there is a proline in the middle of helix G in MCAD (Pro257 is conserved in all mammalian MCADs; pig MCAD has an additional proline, Pro258). This proline causes helix G to bend away from the substrate, making its cavity deeper in comparison to SCADs, i3VD and GD, which do not contain the proline. It is interesting to note that the insertion in helix E that makes shallower cavities in SCAD, i3VD and GD is also missing in the human LCAD sequence.

Another interesting observation is that propionyl-CoA is a poor substrate with bovine SCAD, though it is only one methylene group shorter than the optimal substrate, butyryl-CoA. The k_{cat} of propionyl-CoA with bovine SCAD is 0.1% of that with butyryl-CoA, and in fact propionyl-CoA acts as a suicide inactivator by forming an adduct with FAD, having a partition ratio of 4.3 : 1 between productive catalysis and the suicide inhibition [22]. The structural basis for this unexpected inhibition is not obvious at present.

Oxygen reactivity in the bacterial SCAD (bSCAD)

bSCAD from M. elsdenii reacts very rapidly with molecular oxygen compared to its mammalian counterpart [23]. It should be noted that in M. elsdenii, the enzyme physiologically functions as an enoyl-CoA reductase rather than a dehydrogenase (i.e. the reaction works in the opposite direction) and has no obvious need for protection against oxygen, since the organism is an anaerobe. All known mammalian ACADs, except human iBD (which has a phenylalanine), have a tryptophan protecting the dimethyl-benzene side of the flavin ring at its si-face (Trp166 in MCAD). However, the bSCAD has a phenylalanine at the corresponding position, which makes its flavin more exposed to the solvent, which is consistent with the higher oxygen reactivity of the bacterial enzyme. It would be interesting to see whether iBD also has a higher oxygen reactivity compared to other mammalian ACADs.

The acyl-CoA binding cavity of iso(3)valeryl-CoA dehydrogenase (i3VD)

The structure of human i3VD reveals that, again, the overall polypeptide fold and its substrate-binding mode are very similar to that of MCAD [6]. As in the case of SCAD, the acyl-CoA binding cavity is shallower than that of MCAD (see above), but it is also wider where the C3-C4 atoms of the substrate bind than the same region in either MCAD or SCAD. This optimizes binding of the C3-branched, iso(3)valeryl moiety of the thioester substrate. The specific activities of i3VD with butyryl-CoA and hexanoyl-CoA as substrate are only 20% and 15% of the activity for isovaleryl-CoA, respectively [24]. This C3-branched chain specificity comes from the absence of a bulky residue, tyrosine, at position 374. This tyrosine is conserved in all known acyl-CoA dehydrogenases except iBD, which has a leucine (also a bulky residue) at the corresponding position. Figure 5C shows the binding cavity of i3VD with iso(3)valeryl-CoA modeled into the site. The lack of the tyrosine side
Chain allows the C3-methyl group of the substrate to fit snugly in the cavity provided by the absence of the phenol ring. This arrangement of the -C1-C2-C3- portion of the iso(3)valeryl moiety is ideally suited to allow the pro-R C2 proton to be abstracted by the catalytic base, Glu254, and hydride transfer from C3 to the flavin. On the other hand, i2VD and iBD, both of which are specific for C2-branched substrates, can tolerate a bulky residue (tyrosine in i2VD and leucine in iBD) at this position. However, the structural basis for the C2-branched substrate specificit will require the structure determination of i2VD, iBD, or both.

Catalytic residue in long chain acyl- and iso(3)valeryl-CoA dehydrogenase

Although chemical modification [25], crystallography [4] and mutagenesis studies [26,27] firmly established Glu376 and its homologs as the catalytic residue in MCAD and SCADs, this residue is not conserved in LCAD and i3VD. At the corresponding position, both rat and human LCAD and rat i3VD have a glycine, whereas human i3VD has an alanine. Molecular modeling followed by site-specific mutagenesis strongly suggests that Glu261 located in helix G of LCAD is the catalytic residue [28]. Crystal structure analysis of human i3VD has confirmed that, indeed, Glu254 in i3VD (corresponding to Glu261 of LCAD) is the catalytic residue [6]. Although Glu254 in i3VD is more than 100 residues away from Glu376 in MCAD in the primary sequence (Fig. 1), these two residues are topologically conserved in the three-dimensional structure (Fig. 4) and carry out exactly the same chemistry of catalysis. Furthermore, a double mutant of human MCAD, in which its catalytic base location has been changed to that of LCAD (Glu376Gly/Thr255Glu), has been studied by biochemical and crystallographic methods [5,20]. The resulting enzyme, medium long chain acyl-CoA dehydrogenase (MLCAD) has 20% of the activity of MCAD with octanoyl-CoA, and 25% of the activity of LCAD with dodecanoyl-CoA as the substrate. This, together with the three-dimensional structure of MLCAD, provides further evidence that Glu255 can replace Glu376 as the catalytic residue in the structural frame of MCAD. However, the structural basis for the observed change in the substrate chain length specificity, i.e. a shift toward the longer chain substrates is not clear at present. A complete structural analysis of LCAD will probably shed light in this regard. Similar mutational

Fig. 5. Stereo views of the binding cavities of (A) rat SCAD, (B) bacterial SCAD and (C) i3VD. For clarity, only the binding cavities for the fatty-acyl moiety of the substrates are shown. The surfaces of the ‘rim’ of the cavities are very similar to that of MCAD shown in Fig. 3. The orientation of each molecule is rotated approximately 120° about the y-axis from that shown in Fig. 3. The cavities of both rSCAD (A) and bSCAD (B) are large enough to comfortably accommodate hexanoyl-CoA – the last two carbon atoms shown with smaller, pale gray balls. The ‘base’ of the binding cavity of i3VD is wider due to the lack of a tyrosine at position 374, and the a-end of hexanoyl-CoA (smaller, pale gray balls) can bind to i3VD in two different conformations (C).
studies have also been carried out with i3VD [24], SCAD [27], and i2VD [29], demonstrating that the catalytic residues, Glu254 in i3VD, Glu368 in SCAD, and Glu381 in i2VD are homologs of Glu254 in LCAD and Glu376 in MCAD. These results indicate that the catalytic residue in ACADs can be placed either at 376 as in MCAD (or its equivalent in other ACADs) or at 261 as in LCAD (or 254 in i3VD).

There are many families of distantly related enzymes in which different functional groups are not conserved in the primary sequences, but where catalytic atoms are in the same position spatially [30]. However, the family of ACADs offers the only known example of a mechanistically essentially identical series of enzymes in which the catalytic residue is not conserved in the primary structures. It also serves as a reminder that any conclusions drawn from sequence data alone should be taken with caution. What may have led to such a migration of the catalytic base in the ACAD family? One possibility is that the ancestral gene had glutamates at both locations and that the loss of either one may have led to the evolution of the ACAD family. Another possibility is that the positive charge of Arg94 appears to be involved in the binding of the substrate and in the evolutionary optimization produced the new catalytic residue at a distant position, and that the loss of either one of them led to the evolution of ‘new’, more efficient modern enzymes. A second possibility is that the ‘old’, inefficient enzyme had the catalytic residue at a distant position, and that the evolutionary optimization produced the ‘new’, current enzymes due to a functional group ‘hopping’ to either one of the two current positions. An example of a functional group hopping can be found in the lipase family [31]. A third scenario involves gene (or exon) duplication, in which an exon containing one glutamate duplicated itself at the other location followed by mutations, resulting in migration of the catalytic residue. More complete comparisons of sequences and structures are necessary for a better understanding of the ACAD family lineage.

Structure of glutaryl-CoA dehydrogenase (GD)

GD is unique among ACADs in that it catalyzes not only the α-β dehydrogenation reaction but also decarboxylation of the γ-carboxylate of the substrate, glutaryl-CoA. Preliminary results of structural studies of human GD have been reported [7]. The overall structure is the same as the other known ACAD structures, and the identity of the catalytic base, Glu370, is also confirmed. The most striking difference between the active site residues in GD and those of other ACAD structures is the presence of a positively charged residue, Arg94, at the base of the acyl moiety-binding site. Other ACADs have a neutral residue at this position. A model of glutaryl-CoA fitted into the active site suggests that the γ-carboxylate of the substrate would be within hydrogen bonding distance from the guanidinium group of Arg94. Substitution of Arg94 to glycine or glutamine increases the $K_{m}$ for glutaryl-CoA 10- to 16-fold compared to the wild type enzyme, while the $k_{cat}$ of the mutant enzymes decrease to 2–3% [32]. These mutants are, however, still capable of catalyzing the decarboxylation of glutaconyl-CoA, suggesting that Arg94 is not absolutely required for the decarboxylation reaction. Thus, Arg94 appears to be involved in the binding of the substrate and in the alignment of the glutaryl-CoA substrate for optimum orientation for the dehydrogenation reaction. In addition, the positive charge of Arg94 appears to be involved in the stabilization of the anionic intermediate, crotonyl-CoA anion, during catalysis. The exact mechanism and the structural basis for the decarboxylation reaction must await a complete structural analysis of the enzyme in complex with substrate/analogue.

Electron transfer flavoprotein (ETF) and its interaction with ACADs

In mammalian mitochondria, the physiological electron acceptor of the ACADs is electron transfer flavoprotein (ETF), which is heterodimeric and contains one FAD and one AMP. In addition to being the electron transfer partner of the ACADs, ETF is also the physiological electron acceptor of two other flavoprotein dehydrogenases that are involved in choline metabolism (sarcosine dehydrogenase and dimethylglycine dehydrogenase). The structure of human ETF reveals that the molecule is comprised of three structural domains [33]. Two domains are from the α-subunit and the third domain is composed entirely of the β-subunit. The FAD lies at a cleft between the two subunits and is somewhat exposed to the solvent. AMP is buried in the interior of the β-subunit and is not involved in the redox reaction, strongly suggesting that its role is purely structural.

Very little structural information is available regarding the interaction between mammalian ACADs and ETF. Stable complex formation between these two flavoproteins has not been clearly demonstrated, although it has been reported recently that ETF forms soluble, relatively stable complexes with ACADs in mitochondria [34]. As a result, to date, only a hypothetical model of the complex of human ETF and porcine MCAD is available [33]. In this model, electrons pass from MCAD to ETF at the si-side of the MCAD flavin ring (Fig. 6A), and the closest distance between the two flavins is about 19 Å (between the two dimethylbenzene rings). It is also possible that there are some conformational changes when the two molecules interact, allowing the two flavins to approach more closely for an efficient electron transfer. More recent studies using small angle X-ray scattering techniques with human and Paracoccus ETF revealed that the ETF molecules are indeed flexible enough to form multiple conformations in solution, strongly suggesting that ETF would adopt different conformations when it binds its electron transfer partners [35]. However, detailed sites and the nature of interaction between the two electron transfer partners must await the structural analysis of a complex between ETF and one of the ACADs.

Peroxisomal acyl-CoA oxidase

Peroxisomal acyl-CoA oxidases (ACOs) are the peroxisomal equivalent of the mitochondrial acyl-CoA dehydrogenases (ACADs). They are flavoenzymes containing one noncovalently bound FAD per subunit and belong to the same superfamily as ACADs [12]. Like mitochondrial fatty acyl-CoA dehydrogenases, ACOs catalyze the initial and rate-determining step of the peroxisomal fatty acid β-oxidation pathway, i.e. α,β-dehydrogenation of acyl-CoA, yielding trans-2-enoyl-CoA in the reductive half-reaction. In the oxidative half-reaction of ACO, however, the reduced FAD is reoxidized by molecular oxygen producing hydrogen peroxide, whereas the reduced FAD of ACADs transfer electrons to ETF, thus providing
Compared to the extensively studied ACADs, structural and mechanistic studies of ACOs have been relatively limited, mainly due to the lack of their three-dimensional structure, which has been obtained only very recently [8]. The mechanisms of the reductive half-reactions of ACO and ACAD are very similar, but their physiological oxidative half-reactions are completely different. For example, reduced ACAD transfers electrons to ETF one electron at a time, whereas ACO transfers two electrons to molecular oxygen. Therefore, ACO and ACAD offer an excellent model system for understanding how these two different oxidative half-reactions are controlled at the molecular level.

**Overall structure of rat ACO in comparison with MCAD**

In rat peroxysomes, two acyl-CoA oxidase isozymes, ACO-I and ACO-II, have been identified, each of them having slightly different substrate acyl chain-length specificities [36]. As the structure of only ACO-II (optimal substrate acyl chain length C14 compared to C10 for ACO-I) is known [8], we hereafter refer to ACO-II as ACO for simplicity. Figure 7A and 7B show the polypeptide fold of an ACO monomer and the overall structure of the dimeric molecule of ACO, respectively. Each subunit of ACO is comprised of four domains: N-terminal α-domain, N-terminal β-domain, C-terminal α-domain I, and C-terminal α-domain II (Fig. 7A). The first three domains correspond to the entire subunit structure of acyl-CoA dehydrogenase, whereas the last domain of ACO, which is composed of the C-terminal 221 residues (C-terminal α-domain II), is not present in the ACAD structures (compare Fig. 7A with 2A; Fig. 1). The ACO dimer without the C-terminal α-domain II (Fig. 7B minus the light brown and grey helices) and the MCAD dimer (Fig. 7C) are very similar to each other. However, a close inspection of the two reveals distinct features in each. The relative orientation of the first two domains (the N-terminal α- and β-domains) with respect to the C-terminal α-domain I differs in ACO from the orientation of the corresponding domains in MCAD by about 13°, making the crevice between these domains wider, which in turn makes the binding cavity for the fatty acyl moiety wider and deeper (see the discussion of the shallower cavity in the SCAD structure, above). This 13° rotation also results in differences in the interaction between the flavin ring and the polypeptide chain of ACO, compared to MCAD (e.g. the number of hydrogen bonds between the flavin and the protein moiety is less in ACO than in MCAD) [8]. The active-site cavity of ACO is ≈28 Å long and 6 Å wide and can accommodate the acyl-chain length of C23, in agreement with the acyl-chain length specificity previously determined [36]. This wider active-site cavity in ACO is accessible not only to substrates with long acyl-chains in the reductive half-reaction, but also to molecular oxygen during the oxidative half-reaction.

**Structural basis for the regulation of oxygen reactivity of ACO and ACADs, and of electron transfer to ETF**

In order to understand the detailed molecular mechanism underlying the reactivity (or absence thereof) of reduced ACO and ACADs toward molecular oxygen or an electron acceptor protein, it is essential to consider the physical/structural and chemical aspects associated with the flavin ring system. The physical/structural aspect concerns how oxygen can physically access the reduced flavin embedded in the protein interior and how oxygen access is structurally ensured or prevented, whereas the chemical aspect concerns how electron transfer from the reduced flavin toward oxygen is chemically enhanced or impeded. In the absence of detailed knowledge of the electronic state of the reduced flavin in ACO with and without bound substrate/product, the following discussion of the oxidative half-reaction of

![Fig. 6. Putative ETF docking site in MCAD and the corresponding site in ACO.](image-url)
ACO is limited only to the physical/structural aspect. The basic architecture of the active site cavity is remarkably similar in ACO and MCAD (here used as the representative of ACADs), reflecting their common mechanism for the reductive half reaction. The polypeptide segments surrounding the flavin ring and forming the active site cavity in the two structures are nearly superimposable (rmsd of 0.46 Å) [8], except for on the side of the pyrimidine moiety. While the pyrimidine side of the flavin ring in MCAD is also covered by its polypeptide, the corresponding side in ACO is exposed to solvent. This difference results in reduced hydrogen bonding interactions between FAD and the polypeptide in the ACO structure compared to MCAD. The solvent accessibility of the reduced flavin in ACO is probably responsible for its oxygen reactivity. In contrast, the entire flavin ring in MCAD is well embedded in the protein interior, and thus oxygen access is physically restricted. Only when the active site of MCAD is vacant, i.e. when no ligand is bound, can the reduced flavin be exposed to molecular oxygen and solvent, resulting in oxygen reactivity, although not as high as that of a typical oxidase. In the studies with the bacterial SCAD, the ratio of oxidase to dehydrogenase activity increases as the size of the CoA analog of the substrate decreases, which is consistent with the idea that the more solvent accessible the reduced flavin is, the higher the oxidase activity becomes [37].

Another structural difference that further reinforces the difference in solvent accessibility can be seen in their quaternary structures. The N-terminal side of the C-terminal α-domain II of ACO resides in the region corresponding to the interface between the two dimers of the MCAD tetramer, thus preventing the ACO dimers from associating with each other to form a tetramer. Therefore, the active site of ACO is only partially protected by the small N-terminal side of the C-terminal α-domain II (Fig. 7B), while the MCAD active site is more fully protected by a much bulkier subunit. This again allows ACO easily accessible oxygen to its active site.

Roberts et al. [33] have postulated that, in the oxidative half-reaction of MCAD, ETF approaches MCAD (Fig. 6A), forming an electron-transfer complex in which electrons are transferred from the si-face of the reduced flavin of MCAD to the oxidized flavin of ETF. Figure 6B depicts the region of ACO corresponding to the proposed docking surface of MCAD to ETF. It is noteworthy that helix S of ACO, the C-terminal end of the C-terminal α-domain II of the neighboring subunit in the dimer, covers the si-face of the flavin ring to be further away from the protein surface, thereby interfering with access of ETF to the surface of ACO. Consequently, although electron acceptors corresponding to ETF are not known in peroxisomes, ACO is protected from forming an electron-transfer complex with an ETF-like molecule, should one exist. Therefore, the structural basis for ACO being an oxidase rather than a dehydrogenase is related to (a) oxygen accessibility to the active-site and the dimeric structure rather than a tetrameric form and (b) the si-face of the flavin ring in ACO being further away from the surface of the molecule than in MCAD due to the helix S, thereby preventing ACO from forming an efficient electron transfer complex with an ETF-like molecule.

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