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MINIREVIEW

Acyl-CoA dehydrogenases
A mechanistic overview

Sandro Ghisla[1] and Colin Thorpe[2]

[1]*Department of Biology, University of Konstanz, Germany;* [2]*Department of Chemistry and Biochemistry, University of Delaware, Newark, DE, USA*

Acyl-CoA dehydrogenases constitute a family of flavoproteins that catalyze the α,β-dehydrogenation of fatty acid acyl-CoA conjugates. While they differ widely in their specificity, they share the same basic chemical mechanism of α,β-dehydrogenation. Medium chain acyl-CoA dehydrogenase is probably the best-studied member of the class and serves as a model for the study of catalytic mechanisms. Based on medium chain acyl-CoA dehydrogenase we discuss the main factors that bring about catalysis, promote specificity and determine the selective transfer of electrons to electron transferring flavoprotein. The mechanism of α,β-dehydrogenation is viewed as a process in which the substrate αC-H and βC-H bonds are ruptured concertedly, the first hydrogen being removed by the active center base Glu376-COO^- as an H^+, the second being transferred as a hydride to the flavin N(5) position. Hereby the pK_a of the substrate αC-H is lowered from > 20 to ≈ 8 by the effect of specific hydrogen bonds. Concomitantly, the pK_a of Glu376-COO^- is also raised to 8–9 due to the decrease in polarity brought about by substrate binding. The kinetic sequence of medium chain acyl-CoA dehydrogenase is rather complex and involves several intermediates. A prominent one is the molecular complex of reduced enzyme with the enoyl-CoA product that is characterized by an intense charge transfer absorption and serves as the point of transfer of electrons to the electron transferring flavoprotein. These views are also discussed in the context of the accompanying paper on the three-dimensional properties of acyl-CoA dehydrogenases.

Keywords: fatty acid β-oxidation; acyl-CoA dehydrogenase; acyl-CoA oxidase α,β-dehydrogenation; mechanisms.

Introduction

Acyl-CoA dehydrogenases (ACADs) constitute a rather large family of flavoproteins, which appears to be still growing in numbers. In fact the last member, ACAD-9, was discovered most recently and has the properties of a very long chain acyl-CoA dehydrogenase (vLCAD) [1]. Anticipating its more precise characterization, we have named it vLCAD2 and have classified it accordingly in Fig. 1. ACADs catalyze the desaturation at positions α,β (α,β-dehydrogenation) of various CoA-conjugated fatty acids that stem from either the β-oxidation cycle or amino acid metabolism. In the process, two reducing equivalents are generated that are transferred to electron transferring flavoprotein (ETF) and from this to the respiratory chain via ETF dehydrogenase. The combination of these processes is depicted in Fig. 1.

α,β-Dehydrogenation is a key enzymatic activity in β-oxidation. However, although the ACADs have the lowest activity of the β-oxidation enzymes by several orders of magnitude and will therefore have a high control strength with respect to pathway flux, their overlapping chain-length specificities means that individual ACADs may not be rate-limiting for every turn of the cycle. β-Oxidation has also received considerable attention in the context of genetic defects and their relation to the 'sudden infant death' syndrome (SIDS) and to sudden, unexpected child death as discussed in the accompanying article by Gregersen *et al.* [4]. ACADs transfer electrons specifically to ETF (Fig. 1), and the mammalian enzymes are characterized by a very low rate of reactivity with molecular oxygen. This contrasts with the behavior of the peroxisomal acyl-CoA oxidases that use dioxygen as terminal acceptor and are discussed in the accompanying article by Kim & Miura [5].

There are now a great number of ACAD homologs in the sequence databases. Many of these are acyl-CoA oxidases of peroxisomal origin [6–10]. Others are involved in antibiotic biosynthesis [11–13] or in stress-responses [14,15]. Recently, nitroalkane oxidase, an enzyme that catalyzes the oxygen-dependent conversion of nitroalkanes

Correspondence to S. Ghisla, F. Biologie, Universität, PF 5560, M644, D-78457 Konstanz, Germany.
Fax: + 49 7531 884161, Tel.: + 49 7531 882291,
E-mail: sandro.ghisla@uni-konstanz.de

Abbreviations: ACAD, acyl-CoA dehydrogenase; CT, charge transfer; ETF, electron transferring flavoprotein; GD, glutaryl-CoA dehydrogenase; KIE, kinetic isotope effects; i3VD, isovaleryl-CoA dehydrogenase; i2VD, 'branched chain' acyl-CoA dehydrogenase; iBD, isobutyryl-CoA dehydrogenase; LCAD, long chain acyl-CoA dehydrogenase; MCAD, medium chain acyl-CoA dehydrogenase; SCAD, short chain acyl-CoA dehydrogenase; vLCAD, very long chain acyl-CoA dehydrogenase.

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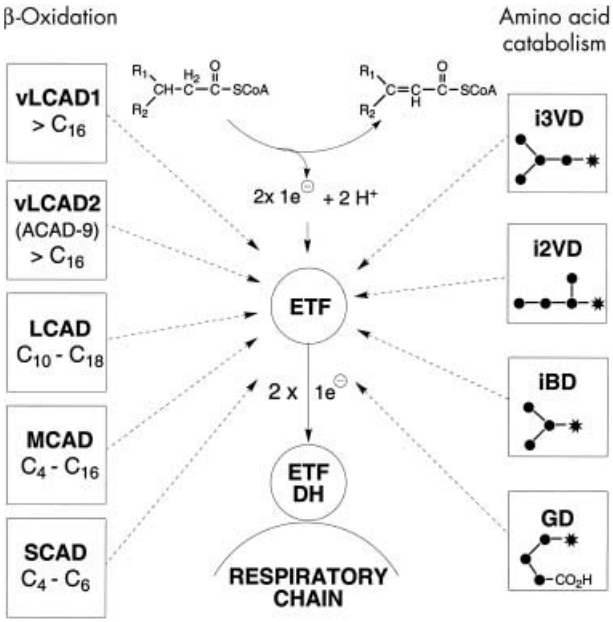


Fig. 1. Enzymes involved in the α,β-dehydrogenation of acyl-thioesters. The fatty acid chains occurring in β-oxidation (left) are usually even-numbered straight chains of variable length. In the structures of those derived from amino acid metabolism (right) '●' indicates saturated C-centers and ✷ (–C = O)-S-CoA. Whether LCAD is better located into the β-oxidation subfamily, or into that involved in amino acid metabolism is still unclear. Recent evidence indicates that it plays an important role in the β-oxidation of medium-chain and long-chain 2-methylacyl-CoAs [2] and of unsaturated fatty acid thioesters [3]. Note the central role of ETF and ETF-dehydrogenase (ETF-DH) in delivering electrons to the respiratory chain.

to the corresponding aldehydes, with release of nitrite and hydrogen peroxide, has been shown to be a member of the ACAD family of proteins [16,17]. There are even homologs which oxidize dibenzothiophene without a requirement for CoA, and with a totally unrelated chemistry [18,19]. Of all these enzymes, medium chain acyl-CoA dehydrogenase (MCAD) remains the best-understood member of the family from a mechanistic viewpoint and will be the focus of much of the discussion below. With few exceptions, all these FAD-dependent enzymes share the essential aspects of the chemistry of activation and subsequent oxidation of acyl-CoA thioesters. This article cannot provide comprehensive coverage of this large area and readers may also want to consult earlier reviews of the literature [20–22].

At the outset, we wish to acknowledge that the pioneering role in the study of this α,β-dehydrogenation reaction was that of Helmut Beinert. Beinert and colleagues, more than 40 years ago, purified MCAD to apparent homogeneity, and made the first mechanistic proposals (reviewed in [22]). Those who have actually read these early papers cannot fail to be impressed by their clarity and rigor. This early work has stood the test of time remarkably well. We summarize below additional mechanistic insights gained largely over the past two decades involving chemical and biochemical methods, molecular genetics and X-ray crystallography.

Overall mechanism

As with most related flavoproteins, ACADs function as an overall 'two-reaction' process represented by:

$$\begin{aligned} E{\sim}Fl_{ox} + SH_2 &\overset{(a)}{\rightleftharpoons} E{\sim}Fl_{red}H^- + S + H^+ \\ E{\sim}Fl_{red}H^- + Acc_{ox} &\overset{(b)}{\rightleftharpoons} E{\sim}Fl_{ox} + Acc_{red}H^- \end{aligned} \quad (1)$$

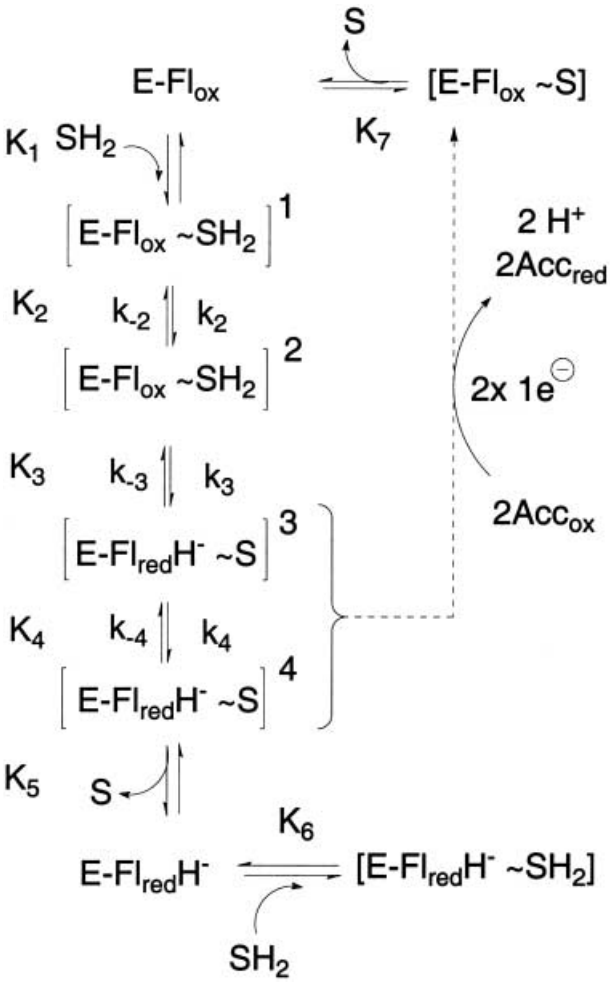
In (a), the 'reductive half-reaction', substrate (SH_2) is dehydrogenated and the resulting redox equivalents are transferred onto the enzyme-bound oxidized flavin (Fl_{ox}) to generate the reduced species ($Fl_{red}H^-$) and product (S). In the present case (a) corresponds to α,β-dehydrogenation of substrate. In (b), the 'oxidative half-reaction', electrons are transferred to the acceptor ETF (Acc_{ox}) regenerating starting enzyme. These two 'half-reactions' will be treated in more detail below.

Kinetic mechanism of the α,β-dehydrogenation

The kinetic sequences required for a description of the 'reductive half-reaction' of MCAD are very complex and are not addressed in detail here. Scheme 1 depicts the minimal sequence (involving equilibria $K_1 - K_7$).

Acyl-CoA ligands bind, in general, quite tightly to the various redox states of MCAD [22,24–28]. Formation of the encounter, or Michaelis, complex $[X]^1$ via equilibrium K_1 (Scheme 1) is fast and probably never rate limiting [29]. Clear evidence for a two-step binding event including a transformation of $[X]^1$ into a second complex $[X]^2$ comes from rapid-reaction studies using chromophoric substrates and products [30,31]. The formation of the initial encounter complex $[X]^1$ is signaled by the rapid perturbation of the flavin chromophore typically induced upon binding of most acyl-CoA ligands.

The rearrangement of $[X]^1$ into $[X]^2$ is substantially rate-limiting with 'fast' substrates such as C_8-CoA [21,29]. This reaction remains poorly understood with native enzyme and physiological substrates because of the subsequent redox reaction and the lack of unique spectral signals for the isomerization step. However, isomerization has been studied using abortive complexes between oxidized enzyme and chromophoric enoyl-CoA product analogs, including those with heteroatom [24,31,32], dienoyl- [33] and aromatic functionalities [30,34]. Isomerization is accompanied by a marked polarization of these chromophores (see later). Further, studies with 4-thia-enoyl-CoA analogs showed that polarization is accompanied by proton uptake as the pK of the catalytic base is elevated markedly from ≈6.0 (in free MCAD [35]) to ≈9.2 in the product complex [31]. The next step (K_3) encompasses the chemistry of α,β-dehydrogenation; k_3 corresponds to the transfer of two redox equivalents to the flavin, i.e., to its reduction, and is rate limiting with many poor substrates. K_3 is fully reversible, and its equilibrium position can vary widely depending on the nature of the substrate. Species $[X]^3$ is the complex of enoyl-CoA with reduced enzyme and owes its blue-greenish color to a charge–transfer interaction with an absorbance maximum typically around 570 nm [36].



Scheme 1. Minimal kinetic scheme for catalysis by MCAD as an example for the general case of ACADs. Steps K_5 and K_6 relate to conditions in the absence of electron acceptors. Reactions occurring in the presence of acceptors (Acc) such as ETF or the ferricenium ion (a convenient artificial electron acceptor of the acyl-CoA dehydrogenases [23]) proceed via the dotted lines (right hand side). E: ACAD/MCAD; Fl_{ox} and Fl_{red}: oxidized and reduced flavin cofactor; SH_2: acyl-CoA substrate; S: acyl-CoA enoyl product; k: single kinetic steps; K: equilibria. $[X]^n$ denote various complexes. Note that the H^+ balance has not been formalized. See text for detailed explanations.

In the absence of an electron acceptor, such as ETF, $[X]^3$ is converted into a further complex, $[X]^4$ and then is followed by product release in step K_5. Product binding/release, is strongly in favor of $[X]^4$ because the overall binding of 'good' products to E~Fl_{red} is extremely tight [22,28,37]. The underlying reason for this tight interaction is the shift of the internal equilibrium, consisting of the species $[X]^1$, $[X]^2$, $[X]^3$ and $[X]^4$, towards the latter ones in order to promote catalysis. This is equivalent to a modification of the microscopic redox potential described by step K_3. A careful dissection of the roles of acyl-CoA ligand and enzyme in the apportionment of this effect has been made by Stankovich and coworkers [38–40]. Interestingly, chemical modification of residues in the active site region can markedly shift the internal redox equilibrium [37].

In the absence of electron acceptors product (S) is released, though at a relatively slow rate (K_5) [29]. The resulting, free reduced MCAD can then bind excess substrate (SH_2; via K_6) the corresponding complex that is devoid of CT transitions. This explains the slow conversion into colorless reduced enzyme forms that can be observed in the presence of large excesses of substrate [22,29,41]. Finally, any free enoyl-CoA product can bind to unreacted oxidized enzyme (K_7), contributing additional complexity to the overall distribution of species. It is remarkable that almost all of these equilibria were incorporated into Beinert's description of the reductive half-reaction more than 40 years ago [22]. Moving to the oxidative half-reaction, electrons are transferred one at a time to ETF, or one of several artificial electron acceptors (see below). Transfer of electrons to acceptors is at the stage of $[X]^3$ or $[X]^4$. Product P is then released via K_7 to conclude the catalytic cycle.

Chemical mechanism of the α,β-dehydrogenation, paradigm

From a chemical point of view the α,β-dehydrogenation in the acyl-CoA dehydrogenases (and, e.g. in succinate and dihydroorotate dehydrogenases) are distinguished from the majority of other dehydrogenations in that it involves the rupture of two kinetically stable C-H bonds. In most other cases, at least one hydrogen is linked to a heteroatom and is kinetically labile. Further, α,β-dehydrogenation occurs with the concomitant transfer of a hydride equivalent to the enzyme-bound oxidized flavin acceptor.

The chemical mechanism of α,β-dehydrogenation as catalyzed by ACADs and related enzymes is depicted in Fig. 2.

The salient points of this reaction can be summarized as follows: (a) dehydrogenation occurs in a '*trans*', R,R mode; (b) fission of the two C-H bonds occurs concertedly; (c) the αC-H hydrogen is abstracted as H^+ by the active center base Glu376-COO^-; (d) the βC-H hydrogen is transferred as a hydride to position N(5) of the flavin; (e) the substrate αC-H pK_a is ≈8 (lowered from > 20); (f) the pK_a of

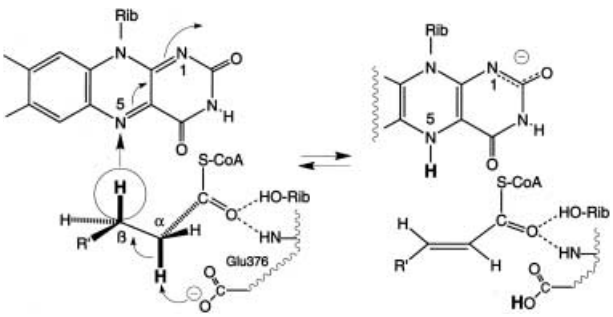


Fig. 2. Chemical mechanism of α,β-dehydrogenation exemplified by MCAD. The figure is a schematic representation of the arrangements of flavin cofactor, substrate and two important H-bonds between Glu376NH and FAD-2′OH and the substrate thioester carbonyl. Rib is the ribityl side chain of the isoalloxazine (derived from the 3D structure and [42]).

Glu376-COOH, the H^+-abstracting base, is ≈9 in the presence of ligand (up from ≈3.5 in the free state and ≈6 in uncomplexed MCAD); (g) the activity and the rate of flavin reduction by substrate are strongly pH-dependent; (h) in the reduced flavin–product-pair a strong CT interaction exists. The above points are discussed below in some detail.

Stereochemistry (a)

The stereochemistry of this reaction (Fig. 2) was reported earlier [43] [44], [45], to be *pro* 2R,3R. This finding was later confirmed by the 3D structure that shows the α, and β-hydrogens to be placed in the conformation required for antiperiplanar reaction [46].

Sequence of C-H bonds rupture (b)

Depending on the mode by which the two C-H bonds are broken in the α,β-dehydrogenation step (carbanion, radical, hydride mechanisms, as discussed in more detail elsewhere [47]) two extremes can be distinguished: (a) rupture of one bond precedes that of the second and an intermediate with definite life time occurs; (b) the two bonds are broken synchronously in one step and no intermediates occur. This is equivalent to a reaction proceeding via a single, symmetric transition state. However, the profile of reaction (b) could be asymmetric, meaning that stretching/rupturing of one bond precedes that of the second. In this case the reaction would be concerted. This question was approached by studying the deuterium isotope effects on the reductive half-reaction, see Eqn 1(a) and step k_3, Scheme 1. Acyl-CoA substrates were used in which the α-, and β-hydrogens were substituted at either one or at both positions (Eqn 2):

$$H_3C\text{-}\overset{(\beta)}{C}D_2\text{-}\overset{(\alpha)}{C}D_2COS\text{-}CoA$$

$$H_3C\text{-}\underset{H}{\overset{D}{C}}\text{-}\underset{D}{\overset{H}{C}}\text{-}COS\text{-}CoA \qquad (2)$$

Using αD_2butyryl-CoA and βD_2butyryl-CoA primary kinetic isotope effects (KIEs) of ≈2–3 and ≈14 were obtained [48]. With 'fully deuterated' (αD_2 + βD_2) substrate, a large KIE ≈15–28 (at 4 °C [49] and 25 °C [48]) was observed that corresponds to the multiplication of the KIEs found with substrate with either the αD_2 or the β-D_2 isotopic substitution. This is compatible with a reaction proceeding via a single transition state [see (b), above] above and [50–53]). For a reaction to proceed through a definite intermediate [see (a), above] e.g. in the case of α-carbanion formation in a first step, the observed KIE would be intermediate between that of a single deuterated substrate and the sum of both single KIEs. In a recent, theoretical study based on the 'molecular dynamics umbrella-sampling simulations and ensemble-averaged variational transition state theory' the dideuterium KIE of the acyl-CoA dehydrogenase reaction was calculated [54]. The estimated values vary from 4.4 to 75 depending on the model used and are compatible with a single transition state, or with a two step model in which the dip in the free energy profile between the proton transfer barrier and the hydride transfer barrier is very small. A further conclusion from this work is that the energy barrier for the hydride transfer step is higher compared to that for αC-H abstraction, in agreement with the finding of a higher KIE for the former. It should be pointed out, however, that with MCAD, and under conditions excluding turn-over, exchange of the αC-H with solvent borne hydrogen does occur [55,56]. This undoubtedly has to proceed via abstraction of the αC-H as H^+ by Glu376-COO^- to form the α-carbanionic species (Eqn 3):

$$\text{R-CDH-COS-CoA} \underset{\text{MCADH}}{\overset{H^+}{\rightleftharpoons}} \text{R-}\overset{(-)}{C}\text{H-COS-CoA} \overset{D_2O}{\rightleftharpoons} \text{R-CD}_2\text{-COS-CoA} \qquad (3)$$

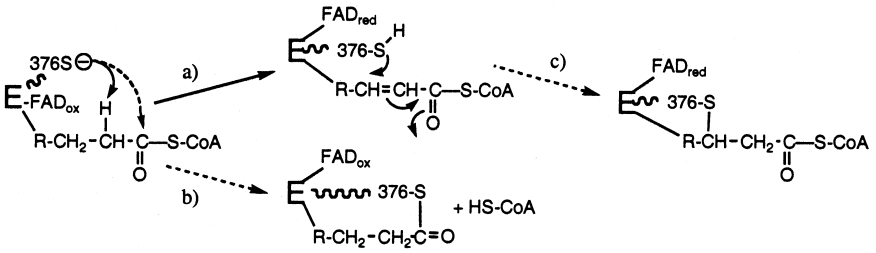
Formation of α-carbanionic species at the active center of MCAD and other selected ACADs can be demonstrated directly using so called chromogenic substrate analogs [35,57] (see also below). The two sets of observations (concertedness of H-bonds rupture and exchange) can be reconciled by assuming that not every event of αC-H bond elongation leads to a concerted expulsion of hydride from position β (committment). In other words, only when the vibrations involving all centers involved in electronic reshuffling are coupled will a productive event occur.

A carboxylate, Glu376-COO-, is the base involved in αC-H abstraction (c)

Within the ACAD family, the catalytic base involved in substrate activation was first suggested to be a glutamate residue by Fendrich and Abeles in their studies of a bacterial SCAD [58]. Using a similar approach, Glu376 was suggested to be the catalytic base in MCAD [59] and this was later established definitively by crystallography [46,60] and mutagenesis [61]. While a carboxylate acts as a base in all ACADs studied so far, the position of the group in the amino acid sequence is not conserved. The ACAD family thus provided an early but not widely recognized example of the migration of a catalytically essential residue within the primary structure of the protein [62]. In MCAD, Glu376 is located on loop JK, while in long chain acyl-CoA dehydrogenase (LCAD) and isovaleryl-CoA dehydrogenase (i3VD) a corresponding Glu is placed at position 255 on the adjacent helix G, the overall topology at the active center being conserved (see discussion in the accompanying paper [5]). A Glu376Asp mutation in MCAD shows ≈5% of the activity of the wild-type (wt) form [63,64]. This difference was attributed to the nonoptimal positioning of the base in the Asp case. Interestingly, the active site of MCAD appears to be very accommodating with respect to the nature of the 376 group in its role in H^+ abstraction.

For example, Glu376Cys-MCAD shows a similar rate of anaerobic flavin reduction compared to wtMCAD when studied with a UV-visible stopped-flow spectrophotometer

(V. Kieweg and S. Ghisla, unpublished obervations). However, no turnover activity is observed, and the enzyme is completely inactive upon further exposure to acyl-CoA substrate. It appears likely that the enzyme is capable of α-H^+ abstraction/catalysis [Eqn 4(a)]. However, two pathways are conceivable that lead to inactivation (Eqn 4(b,c)) that arise from side reactions in which Cys-$SH/^-$ attacks either substrate [transesterification, (b)], or product [Michael-addition, (c)]. In both cases, Cys376 would be covalently modified.



(4)

Of similar mechanistic interest is the observation of substantial activity with the Glu376His mutant amounting to 0.2% of wtMCAD for the reductive half-reaction and turnover [65]. To our knowledge, this is the first case of replacement of a conjugate base with an uncharged one in a biochemical reaction for which substantial activity is retained.

Mode of transfer of redox equivalents from substrate to the flavin (d)

Several possibilities are conceivable for the transfer of electrons from a saturated acyl-CoA substrate to the oxidized flavin. Transfer via covalent intermediates (group transfer), via hydride or via single 1e-steps (radical mechanism) have been discussed [47], the latter going back to an early proposal by Cornforth [66]. A second issue is the locus on the flavin ring that serves in the electron uptake. Both questions have been answered univocally in an experiment in which the flavin in MCAD was replaced with 5-deazaFAD [55]. The latter has two crucial properties: first, in its reduced form, the flavin C(5)-H_2 hydrogens will not exchange with solvent and will retain their stereospecificity [67,68]; second, 5-deazaflavin does not undergo single electron transfer reactions [69]. In the specific case of MCAD, normal substrate does not reduce oxidized 5-deazaFAD, probably for thermodynamic reasons [55]. However, reduced 5-deazaFAD was found to transfer one of its C(5)-H_2 at a rate similar to that of native MCAD to enoyl-CoA [55] (step k_{-3} in Scheme 1). These findings are compatible with direct transfer of the βC-H of substrate as a hydride to the flavin N(5) position. In fact the 3D-structure shows that the substrate βC is located directly above the flavin N(5) position such that the orbital of the βC-H bond would lie in the extension of the flavin LUMO at N(5) as required for a hydride transfer [46] (see also accompanying article [5]).

pK_a of the substrate αC-H (e)

The pK_a of acyl-CoAs has been estimated to be ≈ 20 in the free state [21,42,57,70] and is drastically lowered upon binding to the protein. The αC-H pK_a values of ligands bound to the active center of MCAD have been measured directly using chromogenic substrate analogs of the two following types (Fig. 3).

Note that these redox inactive analogs cannot be dehydrogenated, with reduction of the flavin, due to the lack of a scissile C-H bond at the β-position [42,57,71]. With these ligands their pK_a values can thus be measured directly by following spectral changes as a function of pH. An example is shown in Fig. 4.

The structure of the complex has been solved recently by Satoh *et al.* [72]. The mode of stacking of the flavin with the negatively charged ligand confirm the assignment as charge transfer species, while the authors suggest that the anionic ligand is a transition state analog [72]. The microscopic pK_a of bound 3-thia-octanoyl-CoAs is ≈ 5.2 and was estimated from the pH dependence of the CT extinction coefficient as shown in Fig. 5 [35]. From similar experiments, Tamaoki *et al.* confirmed this estimate using conventional UV-visible and resonance Raman spectroscopy with a value ≈ 5.6 [71]. As the pK_a of the free species is ≈ 16 [57] this translates into a pK_a shift of some 11 units equivalent to 15 kcal·mol^{-1}. With pNO_2-phenyl-acetyl-CoA similar measurements have been performed [35] that indicate a pK_a shift from 14.6 to 5.2, i.e. by ≈ 9.5 units or 13 kcal·mol^{-1}. As detailed elsewhere, a major factor in this pK shift (or activation) is the formation of two tight H-bonds from the FAD ribityl 2′OH and the Glu376NH main-chain proton to the thioester carbonyl oxygen atom [42].

When the 2′OH bond is removed by substituting normal FAD with 2′deoxy-FAD the pK shifts is approximately halved [42] in agreement with the expectation that each of the two H-bonds contributes equally to the interaction and since they both have a ≈ 2.9 Å length [46]. These H-bonds are thus of particular importance in the activation process. A second factor that appears to be relevant in the activation/pK shift is a hydrogen bond between Thr168-OH and the flavin N(5) (see below). To study its effects, this H-bond has

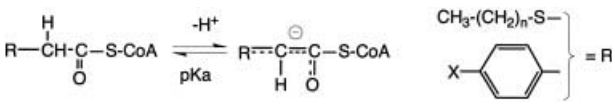


Fig. 3. Types of chromogenic ligands used for the estimation of microscopic pK_a values at the active site of MCAD. (X = -NO_2, -CN, -Ac, etc.) In all cases, unprotonated ligands (left) do not exhibit relevant absorbance above 300 nm, while their anionic forms can be colored. In addition, several anionic ligands can give rise to intense CT transitions such as that depicted in Fig. 4, below.

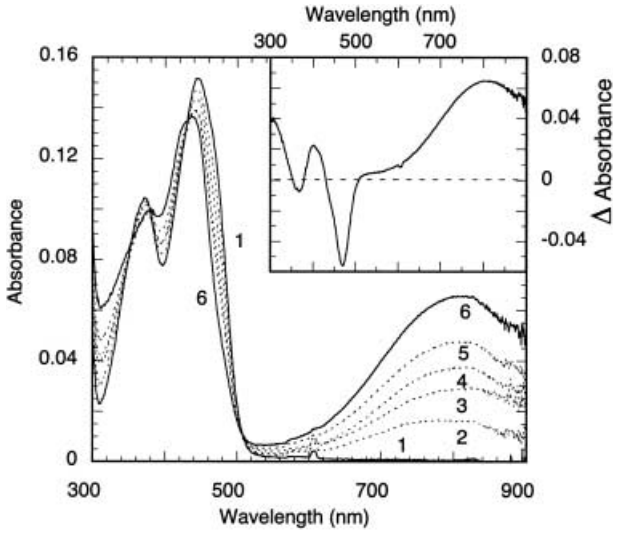


Fig. 4. Spectral effects induced upon binding of 3S-C_8CoA to MCAD. Curve (1) is the spectrum of uncomplexed enzyme, ≈ 1.0 μM in 5 mM Tris/HCl buffer, pH 8.0, in 10 cm cuvettes. Curves (2–6) were obtained upon addition of 1, 2, 3, 4 and 10 μM 3S-C_8CoA (final concentrations, spectra corrected for dilution, estimated apparent $K_d \approx 2.5$ μM). The insert corresponds to the subtraction of curve (1) from curve (6). Note that anionic 3S-C_8CoA itself is not colored, the band in the visible is due to the CT interaction with the oxidized flavin [57,71].

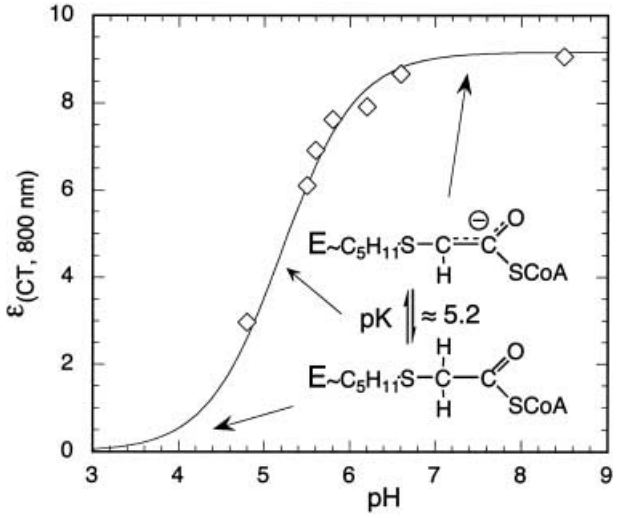


Fig. 5. pH-dependence of the interaction of 3S-C_8CoA with MCAD. The data points were obtained from experiments such as those shown in Fig. 4 and at the pH values shown. The curve is the best fit obtained using the pH equation. The structures denote the anionic form of the charge-transfer donor (top) and the neutral form of bound ligand (bottom).

been removed following two strategies: first, replacement of the native cofactor FAD of MCAD by 5-deaza-FAD [73] leads to an enzyme in which the H-bond in question is not present. Ligands such as 3S-C_8CoA or pNO_2-phenyl-acetyl-CoA bind to this 5-deaza-FAD-MCAD, however, up to pH 11 no αC-H deprotonation of either ligand is observed implying that the pK shift is minor by 5–6 pH units compared to wild-type MCAD (R. Gradinaru, C. Thorpe and S. Ghisla, unpublished observation); second, with the Thr168Ala-MCAD mutant [74], in which the H-bond also is absent, exactly the same effect is observed (R. Gradinaru and S. Ghisla, unpublished observation). While the Thr168-OH hydrogen bond could also affect local conformations and, thus, the reciprocal orientation of ligand and flavin, it appears that a direct influence on the ground state of the flavin–ligand complex, and specifically an increase in the electron deficiency of the flavin N(5), should be considered.

Obviously, the pK of a normal bound substrate cannot be measured directly, because dehydrogenation accompanies deprotonation. However, 3-thiaoctanoyl-CoA appears to be an excellent substrate analog, based both on its electronic and stereochemical properties, and it is thus reasonable to expect that normal substrates would experience a comparable 11–12 unit lowering of the pK upon binding. This leads to an estimation of the substrate αC-H pK_a as 8–9 [31]. This 'activation' can also be assessed by NMR, and Resonance Raman studies using ^{13}C and ^{15}N enriched substrates and cofactors as was demonstrated by the group of Miura and collaborators [71,75–78]. Such experiments are in good agreement with the results presented here. In general terms it should be noted that a decrease in pK is likely to correspond directly to a rate enhancement since both are the consequence of a 'weakening' of the bond in question.

pK_a of the H^+ abstracting base, Glu376-COOH (f)

Based on the expectations for efficient catalysis, the pK values of two groups exchanging H^+ should be roughly comparable [79]. Applied to MCAD this means that the pK of Glu376-COOH should be similar to that of the bound acyl-CoA αC-H (i.e. of the order 8–9). What factors might be responsible for the elevation of the pK from that of a typical aliphatic carboxyl in water? Desolvation, accompanying thioester binding (see accompanying article [5]), likely accounts for much of this effect (e.g. the pK of acetic acid in media of low dielectric can be ≫ 9 [39,80]). While the pK_a of Glu376-COOH cannot be easily measured directly, it has been estimated as ≈ 9.2 in an enzyme-ligand complex, based on the pH-dependent polarization of the substrate analogue 4-thia-*trans*-2-enoyl-CoA [24]. Thus, the pK values of free substrate (≈ 20) and of the catalytic base in the free enzyme (≈ 6) are indeed much more closely matched in the productive enzyme-acyl-thioester complex.

The activity of ACADs is strongly pH-dependent and the dependence, in turn, depends on the substrate chain length (g)

The turnover activities of ACADs and the rate of enzyme flavin reduction that corresponds to α,β-dehydrogenation (step k_3, Scheme 1) are strongly pH-dependent [22,81,82]. This behavior is depicted in Fig. 6 for MCAD and LCAD and selected substrates. Other ACADs that have been studied such as vLCAD1, and SCAD behave similarly [82]. These dependencies reflect apparent pK values that lie in the range pH 6–9. Two aspects are of particular interest in this context. First, it appears that in most cases the activity is low

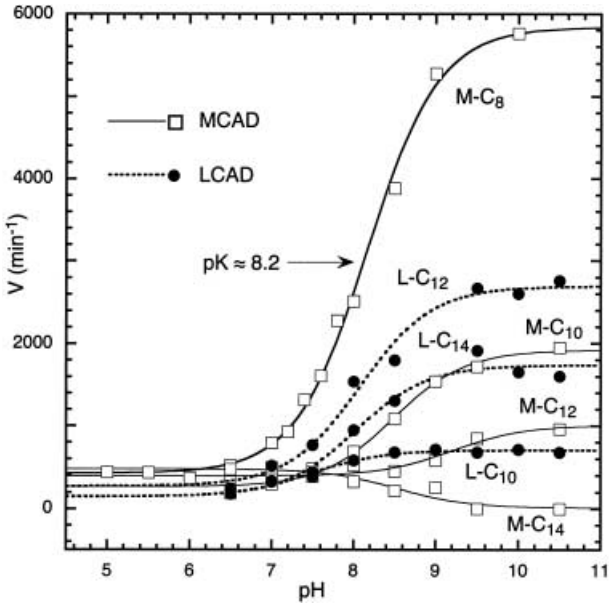


Fig. 6. pH dependence of the activity of MCAD and LCAD and effect of the substrate chain length. The activities were measured with the ferricenium assay [83] using acetate, phosphate, Tris or borate buffers (5 mM) and in the presence of constant 250 mM KCl as electrolyte. M or L indicates either MCAD or LCAD, respsectively, and the suffix the substrate chain length.

but has finite values at low pH and increases with pH. The extent of increase is dependent on the length of the chain (Fig. 6). Thus, in the case of MCAD, a maximal difference (low to high pH) is observed with the best substrate C_8-CoA. On the other hand, with C_{14}-CoA (and C_{16}-CoA, not shown) the activity decreases on going from low to high pH! The molecular factors underlying this effect are not clear and are currently under investigation. While there could be a coincidence between the p*K* attributed to Glu376-COOH in MCAD ($\approx$ 8) [31] with those observed with C_8-CoA and C_{10}-CoA, it is clearly impossible that the ionization of the H^+ abstracting base alone is at the origin of the dependence observed with C_{14}-CoA as with the latter the profile is opposite to expectation. In addition, with SCAD, MCAD and LCAD there is a 'linear' increase of the observed (apparent) p*K* values with the length of the substrate chain (not shown), the slope of the dependence being maximal with SCAD and diminishing progressively for MCAD, and LCAD, while for VLCAD1 no relevant pH effect is found [82]. At present, it can only be speculated that the observed effects result from a combination of factors, the ionization of H^+ abstracting base and the changes in polarity/dielectric induced by binding of the substrate chain being prominent ones.

Role of charge-transfer complexes (h)

ACAD family members participate in a rich diversity of charge-transfer complexes. Beinert showed that earlier suggestions, that the strong green color of SCAD was due to copper, were incorrect [22]. In fact the green color that accompanies a range of natural and recombinant ACADs is due to a tightly bound CoA-persulfide that serves as a charge transfer donor to the oxidized flavin [84]. In general, many CoA ligands that carry a negative charge or an electron-rich functionality at position α/β will show a CT interaction when this functionality is placed on top of the oxidized flavin plane near the C(4a)-N(5) position (see below). A striking example of this type of donor to oxidized flavin CT interaction is shown in Fig. 4. When the electronic distribution is opposite, i.e., the flavin is reduced and electron rich, and complexed with an electron deficient ligand, such as enoyl-CoA, a CT band centered about 550–650 nm is observed. Thus, substrate-reduced ACADs often show a bluish-green color [20,85]. Insight into the nature of these species starts with studies of the acetoacetyl-CoA complex with SCAD [86]. The molecular and steric prerequisites for formation of MCAD CT-complexes were then studied in detail by the group of Miura [10,72,75–78,87,88]. They can be described in analogy to the interactions shown in Fig. 9, where an orbital of the ligand molecule overlaps suitable orbitals of the flavin at the positions N(5)-C(4a). Specifically, with ACADs the oxidized flavin LUMO at N(5) is particularly electron deficient and accepts redox equivalents in the form of a hydride. When it overlaps with an electron-rich orbital of a partner molecule (ground state electronic interaction) a light induced electron transfer from donor to acceptor might occur (CT transition). Just this orientation was found for the CT-complex of MCAD with the anionic form of 3S-C_8CoA [72]; furthermore the same authors estimate the stabilization energy due to the ground state transfer of charge as ≈ 9 kcal·mol^{-1}. The question whether ground state interactions between uncharged species *per se* play a role in flavoprotein catalysis is, however, open to discussion. It is generally assumed that the energetic components of ground state interaction are weak [89]. On the other hand, the observation mentioned above that the presence of the Thr168-OH-N(5)-flavin hydrogen bridge directly affects the p*K* of the ligand αC-H clearly argues in favor of a direct role. The influence of the flavin redox potential on the rates of substrate dehydrogenation and position of CT-absorption bands could be interpreted in analogous terms.

Redox potentials of ACADs and their modulation

The electrochemical studies of Stankovich and coworkers provide important thermodynamic insights into the behavior of ACADs towards their substrates and products. A clear indication of the thermodynamic modulation required for efficient catalysis comes from the realization that free acyl-CoA substrates (–40 mV [40]) would be unable to significantly reduce the free oxidized enzyme (–145 mV; MCAD) [27]. The large positive shift in redox potential when the enzyme binds substrate/product [40] has led to further studies probing the effects of differing types of ligand on the electrochemical behavior of ACADs [27,39,90,91]. It appears that enoyl-CoA binding is an important determinant in this modulation of the redox behavior of these enzymes, together with ligand polarization, and the desolvation-mediated changes in H-bond strength and p*K* of the carboxylate base [27,31,33,34,39,90,91].

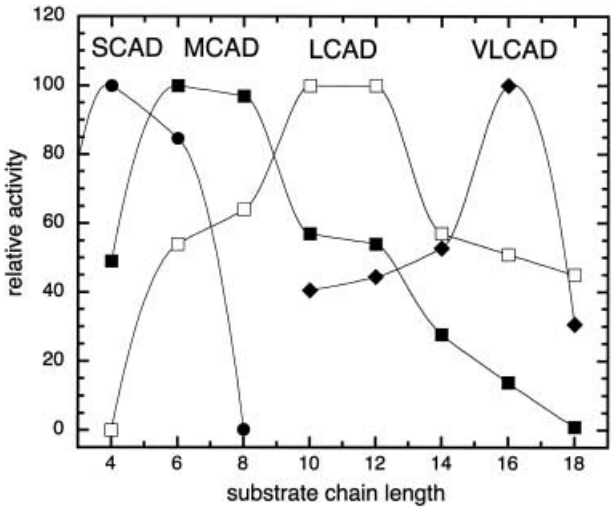


Fig. 7. Dependence of the relative activity of the acyl-CoA dehydrogenases as the substrate chain length is varied. The values have been normalized in order to facilitate comparison. Note that the absolute specific activities of the four enzymes vary considerably.

Substrate chain length specificity

While ACADs share the same basic chemical mechanism, they differ markedly in their specificity towards the 'length' and isomeric substitution of their acyl-CoA substrates. In this context, the two subclasses listed in Fig. 1 behave somewhat differently: the mammalian enzymes acting on straight-chain substrates have comparatively broad specificity that overlap substantially. This is particularly evident with human MCAD and LCAD (Fig. 7), although the rat enzymes appear to have somewhat tighter specificity [92]. Those involved in amino acid metabolism have a much narrower spectrum with a single, strongly preferred, substrate. It should be pointed out that the profiles depicted in Fig. 7 have only qualitative character as branched chain or unsaturated substrates have not been included, and the activity of the dehydrogenases is strongly pH-dependent. Each substrate has a typical, and different pH profile. Thus, depending on the combination of chain length and pH, the relative activities can vary considerably.

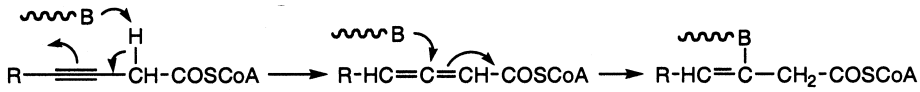
A further factor affecting chain length specificity is the position of the active center base involved in H^+ abstraction [93]. It differs in LCAD and i3VD compared to the other members of the family, and can be interchanged within enzymes [94]. Indeed implementation of the arrangement of LCAD into MCAD leads to an enzyme that has similar activity but a substrate chain length specificity shifted towards that of LCAD [93]. Parenthetically, these studies also suggested that, from a phylogenetic point of view, the two types of enzyme might share a common ancestor that has two Glu residues at the corresponding places [21]. The structural basis for the specificity is discussed in the accompanying article [5].

Inhibitors and inactivators

The modes of inhibition and inactivation of the acyl-CoA dehydrogenases by acyl-CoA thioester analogs are surprisingly diverse. We present below some examples of particular interest.

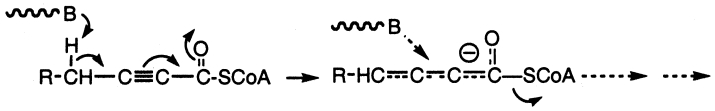
Protein-directed inactivators

3-Alkynoyl-pantetheine or -CoA thioesters are isomerized by the acyl-CoA dehydrogenases to the corresponding electrophilic 2,3-allene with subsequent labeling of the protein [58,95,96] (Eqn 5):



(5)

Unexpectedly, 2-alkynoyl-CoA compounds were also found to be mechanism-based inactivators of the acyl-CoA dehydrogenases [59,97]. They are initially activated by γH abstraction to yield a delocalized enolate of surprising stability [59,97,98] (Eqn 6):



(6)

Using 2-octynoyl-CoA, this anionic species forms a prominent long-wavelength band that decays over many minutes with the release of CoA and the covalent inactivation of MCAD [59,63,97]. Details of the exact sequence of events, which accompany inactivation are still unclear. However, peptide sequencing of MCAD showed that Glu376 is labeled by the 2-alkynoyl moiety and the crystal structure of the inactivated enzyme clearly identifies this residue as the catalytic base [46]. When this carboxylate is located at position 376 (as in MCAD) it appears to have significant flexibility and can therefore abstract suitably activated γ-protons. In contrast, when the base is placed at position 255 on helix G, as in i3VD and LCAD, these enzymes are insensitive to this type of inactivator [99–101].

A completely different type of mechanism-based inactivation of ACADs is provided by compounds A and B in Fig. 7 [102,103]. 5,6-Dichloro-4-thia-5-hexenoyl-CoA (compound A) is a prototype of these compounds and is

activated approximately one in every five turnovers. This leads to β-elimination of a cytotoxic thiolate species and irreversible inactivation of the enzyme. Peptide mapping suggested [104] and crystallography confirmed (J.J. Kim, J.F. Baker-Malcolm and C. Thorpe, unpublished data) that the catalytic base, Glu376, in MCAD is the target of this unusual reaction. The remaining four turnovers release the corresponding *trans*-2-enoyl-CoA product, which then serves as a substrate of enoyl-CoA hydratase [102,105,106]. Consequently, the same thiolate species is released into the mitochondrial matrix from the thiohemiacetal product of the hydratase. 5,6-dichloro-7,7,7-trifluoro-4-thia-5-heptenoyl-CoA (compound B) is a particularly potent inactivator of MCAD and, in addition, can directly inactivate enoyl-CoA hydratase, again via β-elimination of a cytotoxic thiolate fragment [105].

Two other types of thioester-dependent protein-directed inactivation of ACADs have been described. One involves MCAD in the presence of 3-thia-octanoyl-CoA and related ligands. As mentioned earlier, the deprotonated enolate CT donor in these complexes cannot formally reduce the flavin, however, they can undergo 1-electron oxidation by ferricenium with the probable generation of a sulfonium species [107]. Glu376 is the eventual target of this poorly understood oxidative inactivation reaction. Finally, certain 2-halo-acyl-CoA compounds were found to be both substrates and inhibitors of the acyl-CoA dehydrogenases [108,109]. For example, *S*-2-bromohexanoyl-CoA (compound C, Fig. 7) is an affinity label of MCAD with saturable rates of inactivation, strong substrate protection and irreversible inhibition of the enzyme [108]. Again the catalytic base, Glu376, reacts with this class of inhibitory thioesters probably via a simple nucleophilic displacement.

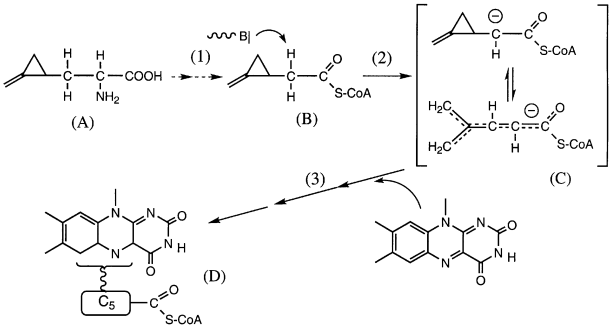
Thioesters that capture the flavin prosthetic group

Diverse acyl-CoA thioesters have been found that target the flavin prosthetic group of ACADs. The first example of covalent capture of the flavin moiety in ACADs is of historical and mechanistic relevance. Hypoglycin (A) (Scheme 2), a toxic amino acid contained in the unripe Ackee fruit is the causative agent of the Jamaican Vomiting Sickness [110,111]. This amino acid is converted to methylenecyclopropylacetyl-CoA (B) the toxic metabolite that was shown more than 20 years ago to be a classic mechanism-based inactivator of SCAD and MCAD [112] (Scheme 2).

In the first step (2) of the inactivation process a carbanion was proposed to be formed, followed by attack of a ring-opened carbanionic species on the flavin [112]. However, for the steps that include ring opening, and formation of covalent adducts (D) with the flavin various routes can be envisaged. Liu and coworkers have observed products consistent with the involvement of radical species in the processing of (B, C) [113]. Analysis of the primary products obtained upon inactivation of MCAD has shown the presence of several adducts that are derived from reaction of the inhibitor (or products arising from its rearrangement) with the flavin [112,114]. Preliminary studies are consistent with points of attachment involving positions 4a, 5 and 6. Similarly, crystallographic data of the inactivation product indicate high electronic density around positions 4a-N(5), they, however, do not allow the attribution of specific structural variants probably reflecting heterogeneity in the structure of the adducts (J. J. Kim, unpublished data, with permission). The failure to identify a unique product of inactivation is not surprising in view of the various possibilities that can be envisaged to result from the interaction of highly instable products such as (C) with the oxidized flavin. The group of Engel has show that a hexyl-substituted analogue of MCPA-CoA is an effective inhibitor of both medium and long chain acyl-CoA dehydrogenases [115].

Another compound, structurally related to MCPA-CoA, spiropentanoyl-CoA (Fig. 8, compound D) inhibits the acyl-CoA dehydrogenases with a component of irreversible inactivation and bleaching of the flavin prosthetic group [116,117]. Cyclobutylacetyl-CoA also irreversibly inactivates the SCAD with apparent reduction of the isoalloxazine ring [118]. Surprisingly, SCAD is inactivated by propionyl-CoA in a reaction that leads to formation of an N-5 flavin adduct [119]. The latter that can be released on denaturation of the enzyme to yield a stable acrylyl-CoA flavin adduct radical [120]. The mechanism of this reaction is still unclear.

While most mechanism-based inactivators are effectively irreversible, ACADs provide a number of examples of



Scheme 2. Simplified sequence for the inactivation of SCAD and MCAD by methylenecyclopropylacetyl-CoA (B). Step (1) encompasses the processes involved in the degradation of hypoglycin (A). The box labeled C_5 in structure (D) represents the product of rearrangement of the methylenecyclopropane that is eventually linked covalently to the flavin ring. See text for details.

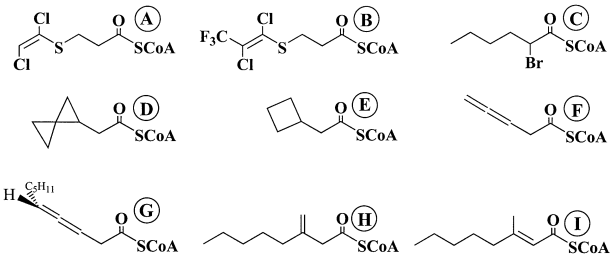


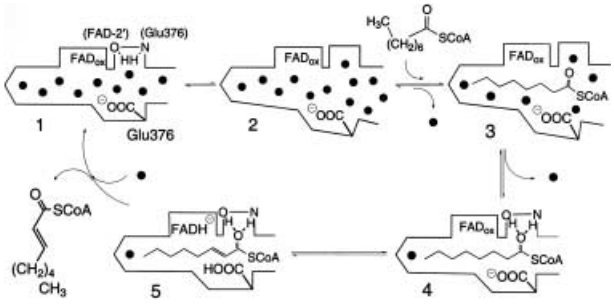
Fig. 8. Selected thioester inhibitors of the acyl-CoA dehydrogenases. Compounds: A, 5,6-dichloro-4-thia-5-hexenoyl-CoA; B, 5,6-dichloro-7,7,7-trifluoro-4-thia-5-heptenoyl-CoA; C, 2-bromohexanoyl-CoA; D, spiropentanoyl-CoA; E, cyclobutylacetyl-CoA; F, 3,4-pentadienoyl-CoA; G, R(–)-3,4-decadienoyl-CoA; H, 3-methylene-octanoyl-CoA; I, 3-methyl-2-octenoyl-CoA.

reversible inactivation. In all these cases, unsaturated thioesters are activated by enzyme-catalyzed deprotonation with subsequent nucleophilic attack of the enolate on the oxidized flavin. The resulting reduced flavin adducts are stable for seconds to weeks before decaying, with release of an isomerized acyl-CoA thioester and with the regeneration of oxidized enzyme. A prototype for this class of inactivators is 3,4-pentadienoyl-CoA (Fig. 8, compound F) [121]. Abstraction of the pro-R-alpha proton generates an enolate species that, rather than discharging a hydride equivalent to the flavin, forms an N-5 adduct instead. This covalent adduct can be reversed in one of two ways. First, by the dissociation of the 3,4-allene itself in the presence of a tightly binding substrate. Secondly, in the absence of competing ligand via isomerization to the more thermodynamically stable 2,4-dienoyl-CoA.

These studies have been extended recently with the synthesis of a series of longer 3,4-allenes such as *R*(–)-3,4-decadienoyl-CoA (Fig. 8, compound G) [122]. The *R*-isomer at C-5 is inhibitory, whereas the *S*-enantiomer is isomerized, apparently directly, to the conjugated 2,4-diene. A crystal structure of the inactivated enzyme [122] confirms the earlier suggestion [121] that these allenic adducts are attached at the N-5 position of the flavin. Further, the structure explains the observed stereochemistry of inactivation. Somewhat surprisingly, adducts formed from the pantetheine thioesters of these 3,4-allenes are more kinetically stable than their full-length CoA counterparts [121,122]. Again, the crystal structures of the -pantetheine and -N-acetyl analogs provides a rationalization, with a dramatic change in the coordination environment of the thioester carbonyl between full length and truncated derivatives [122]. Additionally, the kinetic stability of these adducts is strongly influenced by changes in the size of the alkyl substituent at C5 [122]. Finally, compounds H and I represent another class of unsaturated compounds, which, upon activation, form reversible adducts with the flavin of MCAD.

Reductive half-reaction – a model for catalysis

Scheme 3 depicts the reductive half-reaction, incorporating a number of the mechanistic arguments outlined above.



Scheme 3. Schematic working model depicting the major events accompanying substrate binding and catalysis in MCAD. Glu99 of the active center (positioned at left hand side end of the cavity) has been omitted for clarity (adapted from [31]). • denotes H_2O molecules.

The active site of MCAD is represented as an elongated opening containing the key elements necessary for catalysis. These are a hydrophobic lining, the FAD cofactor, the active center base and the two specific H-bond acceptors shown. In the uncomplexed enzyme, the cavity is filled with a string of ordered water molecules [5,46] (1). Breathing motions of the cavity (2) allow for the accommodation of the hydrophobic end of the substrate and for the expulsion of some water molecules (3). Formation of (3) might correspond to that of complex $[X]^1$ in Scheme 2. The precise docking of the thioester carbonyl into the cavity containing Rib2′OH and Glu376NH in a hydrophobic environment leads to formation of two tight H-bonds, and to strong polarization of the thioester function. The role of this locus can be compared to that of the 'oxyanion hole' first described in protease catalysis [123,124]. Triggered by this event, or concomitantly with it, the residual water molecules are expelled leading to a marked decrease in dielectric at the site. This, in turn, destabilizes Glu376-COO^- increasing its pK to ≈ 9 (4). Theoretical studies suggest that a carboxylate oxygen of Glu376 can form a surprisingly strong H-bond type interaction with the C-H proton about to undergo abstraction and this would position the participants prior to dehydrogenation [125]. In summary, the efficient sequestration of the active site from bulk solvent would ensure that the polarized (and now strongly acidified) thioester would be a facile proton donor for the adjacent Glu376-COO^-. Desolvation minimizes an unproductive reprotonation of Glu376 from solvent water that would otherwise short-circuit catalysis [31]. Thus, a strong base is created along the reaction coordinate and is not present in the initial encounter complex.

The system is now optimized for catalysis and relaxes with the flow of negative charge from carboxylate, through the substrate α,β-bonds to the pyrimidine ring of the flavin (pK < 7 [39]). The mode of electron flow and orbital rearrangements is depicted schematically in Fig. 9. Additionally the Thr168 hydrogen bond to the oxidized flavin N(5) could enhance the electron deficient character of the latter.

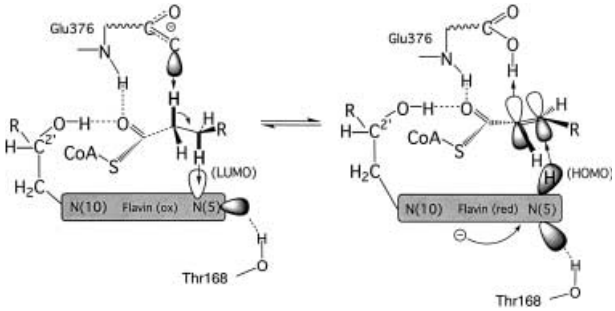


Fig. 9. Reciprocal orientation of orbitals and functions during substrate dehydrogenation. Note that the flavin is viewed along its plane and that substrate is 'sandwiched' between it and the Glu376-carboxylate. One of the main components of the driving force of the reaction is assumed to be the pK increase of Glu-COOH due to the increase in hydrophobicity accompanying expulsion of water from the active site (see also Scheme 3). Further factors are assumed to be stronger H-bonds to reduced, desolvated flavin, in particular that from Thr168 to N(5), and contributions from the reduced flavin~enoyl-product CT complex.

The oxidative half-reaction

As outlined in Fig. 1, ETF is the obligatory acceptor for all ACADs and plays a central role in collecting electrons to funnel them into the respiratory chain via ETF dehydrogenase. Both ETF and ETF dehydrogenase were purified initially and characterized by Beinert and coworkers [22,126]. Most ETFs are heterodimers [127] with a single molecule of FAD and a redox-inactive AMP moiety [128,129] that may be a vestige from a primordial ETF diaphorase [127,129]. The interflavin redox reaction between acyl-CoA dehydrogenase and ETF involves obligatory 1-electron steps to two successive ETF molecules [127,130]. As both dehydrogenase and ETF flavin rings are largely buried [21,46,131] they cannot approach close enough to exchange a hydride equivalent.

The relatively weak interactions between ACADs and ETF have been probed by kinetic analyses, protein modification and cross linking studies [127,132–134]. In the absence of co-crystals of dehydrogenase and ETF, Kim and coworkers identified a shallow depression on the dehydrogenase ringed with charged residues as the likely ETF docking surface. Their initial modeling of MCAD/ETF complexes assumed rigid body interactions that placed flavin centers at the rather large distance of about 16–19 Å, considering the almost isopotential reaction [21,40,130] between substrate-reduced dehydrogenase and ETF [131]. More recent small-angle X-ray scattering experiments with human and Paracoccus denitrificans ETF show a dynamic population of conformers [135]. Correspondingly, multiple configurations of dehydrogenase–ETF complexes may exist and attain closer interflavin distances and higher intrinsic transfer rates [135].

Why does reduction of ETF occur at the level of the enoyl-CoA product-complexed enzyme in this compulsory ordered ternary mechanism [136]? First, as acyl-CoA thioesters are comparatively weak thermodynamic reductants of the dehydrogenases (Scheme 2, K_3) [40,137], they shift the redox equilibrium towards products by preferential binding of enoyl-CoA to the reduced enzyme (Scheme 2, K_5/K_4) [22]. This mandates that product remains bound until the ACAD is reoxidized with reduction of ETF. Second, while enoyl-CoA product complexes of the reduced ACADs are poorer thermodynamic reductants than the free reduced MCAD [40,137], such complexes are much faster kinetic reductants [130]. The acceleration of electron transfer from reduced dehydrogenase to ETF is also seen with complexes of a variety of other thioester ligands and when ETF is replaced with the facile artificial electron acceptor ferricenium hexafluorophosphate [83]. Binding thioesters does not lead to large changes in the static conformation of the dehydrogenases [46] but does promote a sizable lowering of the p*K* of reduced flavin species and flavin analogues [26,83,138]. Deprotonation would be expected to increase the kinetic reactivity of reduced flavin on theoretical grounds [21,83]. Thus, thioester binding modulates key aspects of the reoxidative phases of acyl-CoA dehydrogenase catalysis.

Lastly, bound ligand has a critical effect on ACADs from aerobic organisms – it markedly suppresses the oxidase activity of the enzyme in comparison to, e.g., SCAD from the obligate anaerobe *Megasphaera elsdenii* [20,22]. While the related acyl-CoA oxidases [5,139–141] use dioxygen efficiently, reduced MCAD complexed with enoyl-CoA product is essentially devoid of oxygen reactivity. This brings up the question about the molecular factors that determine oxygen reactivity in both classes of enzymes [139,142]. As detailed in more depth in [5,143], at the active centers of the two types of enzymes there is no difference with respect to presence or absence of specific protein functional groups. On the other hand, the active site of oxidases appears to be more open and accessible to solvent, and the binding pocket for the substrate 'tail' could be more flexible. In conclusion, it thus appears that oxygen reactivity of reduced ACADs is not directed by the presence of specific functional groups at the active center. Moreover it is likely that the combination of physical accessibility of dioxygen [144–147] and of solvent [139,142], both of which would be affected by the same constraints, play important roles. The presence of solvent will influence the degree of solvation and stability of intermediates arising from the primary exchange of electrons between reduced flavin and dioxygen and thus affect the rate of this primary step.

Acknowledgements

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